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INFERRING PHYLOGENY USING GENOMIC CHARACTERS: A CASE STUDY USING LAND PLANT PLASTOMES[1]

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Abstract

The large influx of comparative DNA sequence data has led to many new inferences regarding the tree of life. Early branches in the phylogenetic history of major clades are particularly hard to reconstruct due to the phenomena of long branch attraction. Several analyses spanning land plants have utilized data from different genetic markers. Nevertheless, there remains disagreement about the order of branching among the major clades. Some short, deep branches are proving recalcitrant to reconstruction using phylogenetic analysis of DNA sequence data. In this study, we have identified 40 potentially informative genomic characters from the structure of the plastome (comprising gene arrangement, intron, and gene presence/absence characters) that may help to elucidate the branching order at the base of the land plant phylogeny. A parsimony analysis of these data included 18 taxa, with exemplars of charophytes, liverworts, mosses, hornworts, moniliforms, conifers, and angiosperms. It produced ten most parsimonious trees with an RI of 0.91 and bootstrap support > 90% for pines, grasses, and angiosperms. Five putative synapomorphies unite land plants, land plants minus *Marchantia* is supported by a single intron character, and *Anthoceros* plus vascular plants is supported by two putative synapomorphies. These chloroplast genomic data are potentially useful for addressing phylogenetic questions not amenable to solution by means of DNA sequence comparison, as evolutionary changes appear to occur rarely and are complex (i.e., with many possible character states). Their utility will be tested further in the near future, with the much larger number of whole chloroplast genomes currently being sequenced.

Key words: bryophyte, chloroplast, embryophyte, genomics, land plant, phylogeny, plastome.

Some of the most intriguing questions in systematics concern the origin and relationships of diverse, ecologically dominant lineages. Such groups include metazoans, vertebrates, land plants, and flowering plants. These groups are all thought to have undergone an early rapid radiation, perhaps mediated by an ecological release or the development of a key innovation. This period of rapid diversification was followed by long periods of additional diversification, specialization, and extinction (Kenrick & Crane, 1997; Qiu & Palmer, 1999; Bromham, 2003). The rapidity of the early diversification, compared to the length of subsequent time spent following distinct histories, makes phylogenetic reconstruction of the relative branching order of basal nodes in these groups difficult (see Chapman & Waters, 2002). This pattern leaves little evidence of the order of branching due to the inferred short time periods spanned by the deepest branches, leaving few synapomorphies to define subsets of taxa. Therefore, the search has been intense for evidence to give support to phylogenies of these groups.

The large influx of comparative DNA sequence data has led to many new inferences regarding the tree of life. Nevertheless, certain regions of the tree are currently ambiguous or subject to disagreement depending on the different genetic markers used for phylogenetic reconstruction. Early branches in the phylogenetic history of major clades are particularly hard to reconstruct due to the phenomena of long branch attraction (caused by data saturation on some long branches combined with the paucity of useful characters on connecting short, deep internodes; see Hendy & Penny, 1989; Swofford & Olsen, 1990; Mishler, 2000). Several analyses spanning land plants have utilized data from different genetic markers (Hori et al., 1985; Mishler et al., 1992, 1994; Albert et al., 1994; Barnabas et al., 1995; Malek et al., 1996; Kallersjo et al., 1998;

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Qiu et al., 1998; Duff & Nickrent, 1999; Soltis et al., 1999; Nickrent et al., 2000). Most of these analyses show the "bryophytes" (mosses, liverworts, and hornworts; quotation marks indicate inferred paraphyly) forming a paraphyletic group at the base of the living land plants (see Goffinet, 2000, for summary). However, there is disagreement about the order of branching among the major clades (see discussion). Given the apparently rapid early radiation of major land plant lineages, DNA sequence data may not be effective in definitively inferring phylogeny (Mishler, 2000). This is particularly true of studies that use data from only one gene. In such cases, even extensive sampling cannot counteract the effects of extinction and character saturation (see discussion in Soltis et al., 1999). Instead, the use of genomic structural characters may be necessary for this and similar situations.

Studies using RFLPs to map the makeup of the chloroplast plastome found a large structural rearrangement that is a synapomorphy for tracheophytes (vascular plants) minus lycophytes (Raubeson & Jansen, 1992). In addition, a 22 kilobase (kb) inversion in the choloroplast genome was detected in most genera of the sunflower family, Compositae, but is lacking in Barnadesioideae (Jansen & Palmer, 1987), and a 50 kb inversion defines a group of Phaseoleae in the legumes, Leguminosae (Doyle, 1994, 1995).

Because gene rearrangements are unusual events (Raubeson & Jansen, 1992) and presumably can occur anywhere in the plastome, they are viewed as being much less subject to homoplasy than sequence data (Boore et al., 1995; Boore & Brown, 1998; Rokas & Holland, 2000). Therefore, even a few potentially informative characters from the structure of the genome (herein we will call these genomic characters) can be extremely useful in reconstructing phylogenies (Dowton et al., 2002; Gallut & Barriel, 2002). In this regard, rare genomic characters are analogous to significant morphological characters that often define major plant clades (Mishler et al., 1994). For example, Qiu et al. (1998) found that the acquisition of three mitochondrial gene introns supports liverworts as the sister group to the remainder of land plants (embryophytes). Although these represent few characters, they are quite significant given the rarity of such modifications in the organisms studied. Similarly, Williams and Friedman (2002) argued that the presence of diploid versus triploid endosperm in plesiomorphic angiosperms, such as Nymphaeaceae and Illiciaceae, is a character state that may define certain natural groups.

Recent developments in genomics mean that ever-increasing amounts of DNA sequence data *and* genomic structural data will become available in the near future. Several plant nuclear genomes have been sequenced or are in the process of being sequenced. In addition, several laboratories across the country are actively sequencing the chloroplasts and mitochondria of a large number of green plants (e.g., see table at: ⟨http://ucjeps.berkeley.edu/TreeofLife/data_table.php⟩). While the resultant comparative DNA sequence data no doubt will contribute much to phylogenetic reconstruction, short, deep branches could prove recalcitrant to such investigation. Thus, the use of genomic structural data may be instrumental in reconstructing such difficult phylogenies.

The land plants provide an excellent study system for this approach. There is a broad consensus, based on both morphological and molecular data, that land plants comprise a monophyletic group. After appearing in the Ordovician period (Gray, 1993), they underwent a rapid radiation in the Silurian, with most major lineages appearing by the early Devonian (see Kenrick & Crane, 1997). Many of the groups are well defined morphologically, and there is a developing consensus, based on fossil evidence and DNA sequence data, on the arrangement of many of the interior branches of the phylogeny. Most of the main clades (e.g., angiosperms, conifers, moniliforms) have a representative taxon with a complete sequence of the chloroplast genome available. The land plants as a whole are rooted somewhere among the three bryophyte lineages. Nevertheless, there is a great deal of uncertainty concerning the initial branching order at the base of the land plant phylogeny; nearly every possible branching order among the bryophyte lineages and tracheophytes has had some support (Mishler & Churchill, 1984; Garbary et al., 1993; Hedderson et al., 1996; Garbary & Renzaglia, 1998; Nishiyama & Kato, 1999; Karol et al., 2001; see an excellent summary at ⟨http://www.science.siu.edu/landplants/PhylogRelsGen.html⟩). Therefore, land plant phylogeny, with both well-supported (e.g., vascular plants, seed plants) and ambiguous (e.g., the deepest branches in land plants) subclades, provides a suitable subject for evaluating the utility of chloroplast genomic data.

In this study, we have identified potentially informative genomic characters that may help to elucidate the branching order at the base of the land plant phylogeny. In our utilization of gene arrangement, intron, and gene presence or absence characters, we address theoretical and practical issues involved in the phylogenetic analysis of whole genome sequences.

Table 1. GenBank accession numbers and sources of chloroplast gene maps for sampled taxa.

Taxon	GenBank accession No.	Reference
Charophytes		
Chaetosphaeridium globosum (Nordst.) Kleb.	NC_004115	Turmel et al. (2002)
Liverworts		
Marchantia polymorpha L.	NC_001319	Umesono et al. (1988)
Mosses		
Physcomitrella patens (Hedw.) Bruch & Schimp.	NC_005087	Sugiura et al. (2003)
Hornworts		
Anthoceros formosae Steph.	NC_004543	Kugita et al. (2003)
Moniliforms		
Adiantum capillus-veneris L.	NC_004766	Wolfe et al. (2003)
Psilotum nudum (L.) P. Beauv.	NC_003386	Wakasugi et al. (unpublished)
Conifers		
Pinus koraiensis Siebold & Zucc.	NC_004677	Noh et al. (unpublished)
Pinus thunbergiana (Parl.) Franco	NC_001631	Wakasugi et al. (1994)
Angiosperms		
Arabidopsis thaliana (L.) Heynh.	NC_000932	Sato et al. (1999)
Atropa belladonna L.	NC_004561	Schmitz-Linneweber et al. (2002)
Epifagus virginiana (L.) W. P. C. Barton	NC_001568	Wolfe et al. (1992)
Calycanthus floridus L.	NC_004993	Goremykin et al. (unpublished)
Lotus japonicus (Regel) K. Larsen	NC_002694	Kato et al. (2000)
Nicotiana tabacum L.	NC_001879	Kunnimalaiyaan & Nielsen (1997)
Oenothera elata Kunth subsp. *hookeri* (Torr. & A. Gray) W. Dietr. & W. L. Wagner	NC_002693	Hupfer et al. (2000)
Oryza sativa L.	NC_001320	Morton & Clegg (1993)
Spinacia oleracea L.	NC_002202	Schmitz-Linneweber et al. (2001)
Zea mays L.	NC_01666	Maier et al. (1995)

Materials and Methods

The plants examined for this study may be found in Table 1 and include one or more examples of charophytes, liverworts, mosses, hornworts, moniliforms, conifers, and angiosperms. Gene maps were downloaded from GenBank (Table 1) and manually added into a spreadsheet using Microsoft Excel X for Mac (Microsoft Corp., 2001). We aligned the genes among a section of the plastome in the large single copy region at *rpo*A that has one of the longest regions that is invariant across sampled taxa. Sequence alignment was done by hand, with particular attention to regions of putative inversions. Large inverted sections of gene sequences were analyzed in reverse order to facilitate identification of additional gene rearrangements within the inverted region.

Visual inspection revealed that the presence, location, and order of tRNA genes were the most variable elements in the plastome. In order to prevent misalignment, tRNA genes were removed for the initial alignment and subsequently added in their appropriate positions.

Characters were searched for by using basic principles of character analysis originally developed for morphological characters (Mishler & De Luna, 1991). Characters comprised three types: gene rearrangement characters representing inversions of two or more genes in the plastome, gene presence/absence characters representing a gene missing from a particular position in the plastome, and intron presence/absence representing the presence of a particular intron within genes in the plastome. Duplications of genes via inclusion in the inverted repeat (IR) region were included with gene rearrangement characters. Coding of inversion

Table 2. Data matrix and character state assignment. Refer to Appendix I for character state explanations. Characters are binary, with ? representing unknown data.

	10	20	30	40
Chaetosphaeridium globosum	0001100000	0001100100	1100000000	1111101010
Marchantia polymorpha	0001100000	0000000000	1100000000	1111111011
Physcomitrella patens	0001100000	0100000000	1100000000	?11111111?
Anthoceros formosae	1001100000	0000000000	1100001000	1111111111
Psilotum nudum	1001000010	1000000001	1100011000	1111?11111
Adiantum capillus-veneris	1101110010	0000001001	1100001000	1111111111
Pinus koraiensis	?010101010	0000000010	11000?1001	1101111?00
Pinus thunbergiana	1010101110	0000000010	11000?1001	1101111111
Oenothera elata subsp. *hookeri*	1001000011	0010000000	1101111001	0111111111
Oryza sativa	1001000011	0000010000	111?010101	0011110100
Zea mays	1001000011	0000010000	1111010101	0011110100
Spinacia oleracea	1001000011	0000000000	1101011001	0110111111
Calycanthus floridus	1001000011	0000000000	1101011001	0111111111
Arabidopsis thaliana	1001000011	0000000000	0101011011	0111011011
Atropa belladonna	1001000011	0000000000	0101111011	0111111111
Nicotiana tabacum	1001000011	0000000000	0101111011	0111111111
Lotus japonicum	1001000011	0000000000	0001111011	0111111011
Epifagus virginiana	1001000011	0000000000	100101102?1	011111??11

characters was binary and chosen to minimize the number of inversion characters. Introns were located in gene sequences and coded separately. In addition, copies of genes or pseudogenes were coded as present or absent based on synteny (their location in relation to other genes in other taxa in the analysis). In the coding of characters 0 represents absence or a putative plesiomorphy, while 1 represents presence or putative apomorphy. The final comparative alignment is available as an Excel file on the Green Tree of Life website (⟨http://ucjeps.berkeley.edu/TreeofLife⟩).

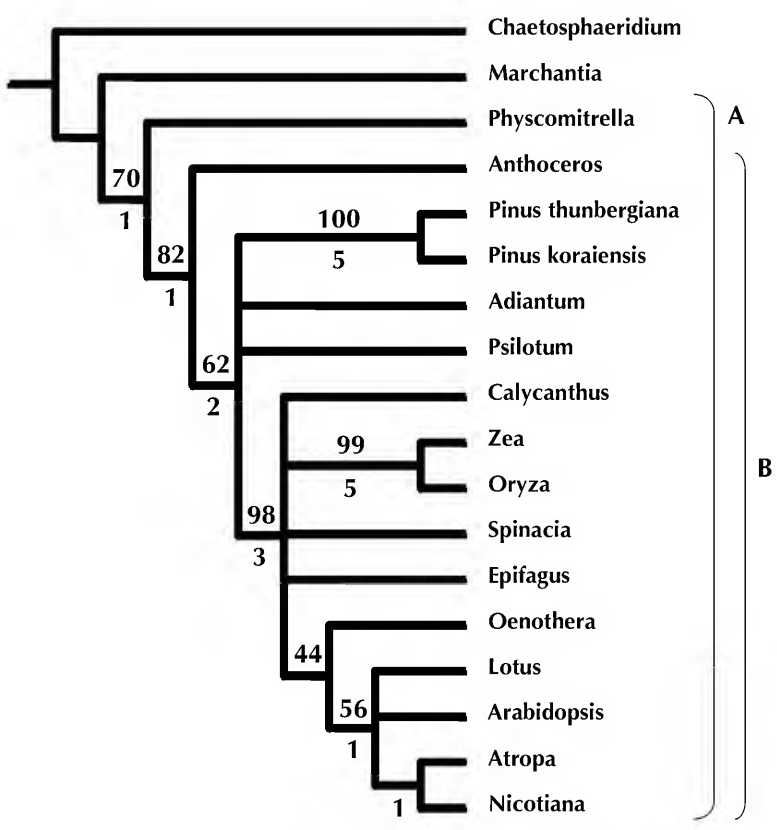

Figure 1. Strict consensus tree of 14 most parsimonious trees. Values above branches represent bootstrap values; values below branches represent decay values. Clade A is all land plants minus *Marchantia*. Clade B is vascular plants plus *Anthoceros*. Bootstrap values greater than 40 are included on figure.

All characters that varied among sampled taxa were included in the data matrix, including autapomorphies, as future sampling will no doubt change some autapomorphic characters into synapomorphies. Forty characters (27 potentially informative) were discovered in all (listed in Appendix 1) and placed in a NEXUS file (Table 2). All analyses were done with PAUP* 4.0b10 (Swofford, 2003). The matrix was analyzed using the branch-and-bound algorithm with the furthest addition sequence setting. The resulting trees were rooted using the charophyte *Chaetosphaeridium* as the outgroup. A bootstrap analysis was performed using a 1000 replicates of heuristic searches employing stepwise addition and TBR branch swapping.

Results

The analysis produced 14 equally parsimonious trees (EPTs; CI = 0.87, RI = 0.91). The strict consensus tree is shown in Figure 1. Of the 40 characters, 13 rearrangement characters, 11 of the gene presence or absence characters, and 4 of the intron characters proved parsimony informative.

High bootstrap values included strong support for a monophyletic *Pinus* (100), monophyly of the grasses (99) and angiosperms (98), and sister relationships between the vascular plants and *Antho-*

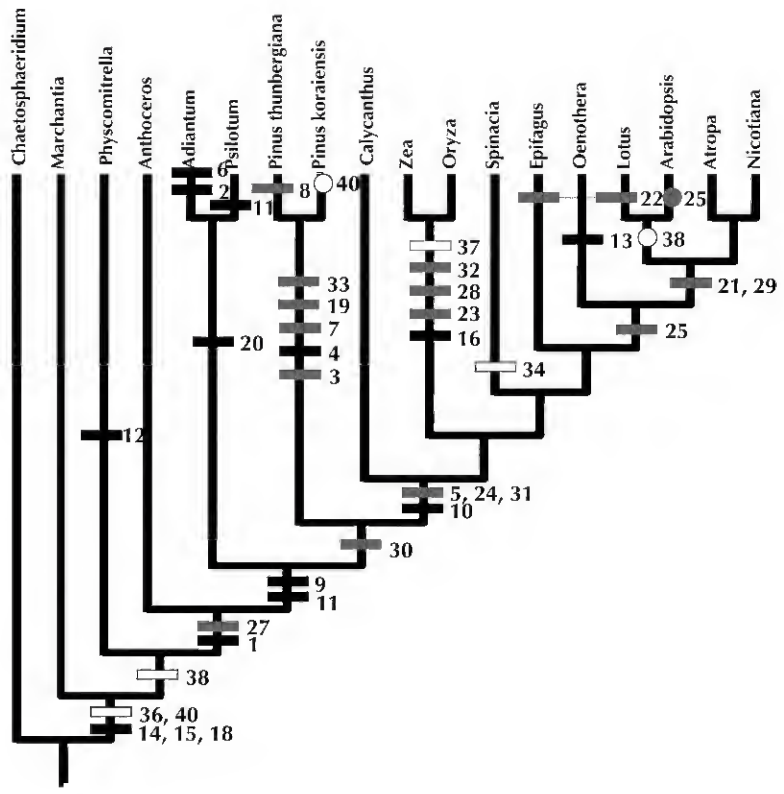

Figure 2. One of fourteen most parsimonious trees with synapomorphies labeled. The number of each character (see Appendix 1) is indicated to the right of each symbol. Black bar = gene rearrangement (including IR region size change); gray bar = gene deletion; white bar = intron loss/gain; circle = inferred reversal. The horizontal dotted line indicates inferred homoplasy.

ceros (82). Resolution and support were lacking for relationships among major lineages of vascular plants, other than the groups mentioned above (see Fig. 1).

Five putative synapomorphies unite land plants, three rearrangement characters (Appendix 1, characters 14, 15, 18) and two intron characters (Appendix 1, characters 36, 40; see Fig. 3). Land plants minus *Marchantia* is supported by a single intron character (Appendix 1, char. 38). *Anthoceros* plus vascular plants share one rearrangement character state (Appendix 1, character 1) and the presence of *rps*12 in the IR_B (Appendix 1, character 27). Vascular plants share the presence of *rpl*21 between *ndh*F and *rpl*32 (Appendix 1, character 30). Angiosperms share the inclusion of *rpl*23 and *rpl*2 in the IR region, as well as three gene presence/absences (Appendix 1, characters 5, 24, 31). Some well-defined groups are supported by putative synapomorphies, such as *Pinus* (Appendix 1, characters 3, 4, 7, 19, 33) and grasses (Appendix 1, characters 16, 23, 28, 32, 37; see Fig. 2).

Discussion

The consistency index for the analysis of land plant genomic data is higher than the average for real data sets (see Sanderson & Donoghue, 1989; Mishler et al., 1994); this indicates a relatively low level of inferred homoplasy for the genomic character set. This is consistent with the expectation that genomic characters are less prone to homoplasious change than DNA sequence characters. Most clades with strong bootstrap support (cf. Fig. 1) originate within groups that have long been recognized based on morphology. These include exemplars from angiosperms, grasses, and pines. The clade (Fig. 1) comprising land plants minus *Marchantia* (the liverwort exemplar in the present study) has an equivocal bootstrap value of 70. However, bootstrap values may not be particularly useful in analyses of this type, in which many branches are supported by few characters. In our data set, the clade "land plants minus *Marchantia*" is supported by one synapomorphy (cf. Fig. 2): an intron loss (Appendix 1, character 38, intron 2 missing from *ycf*3). These two synapomorphies support a topology in agreement with the other studies identifying liverworts as the earliest branch in land plants: studies based on morphology (Mishler & Churchill, 1985) and sequence data from the chloroplast gene *rbc*L (Lewis et al., 1997). In addition, three intron gains (Qiu et al., 1998) in the mito-

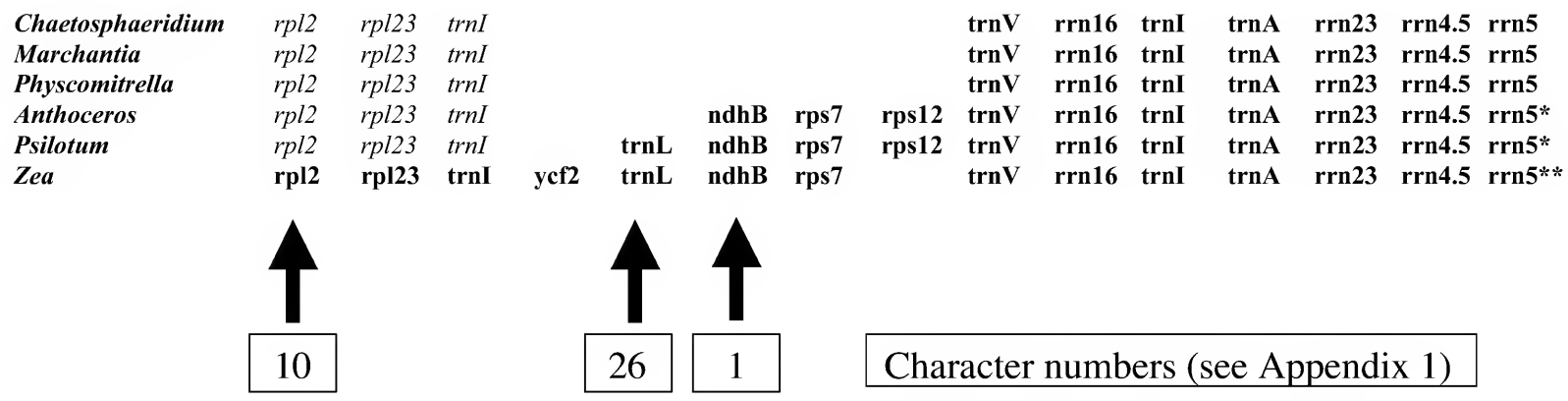

Figure 3. Evolution of the inverted repeat (IR). One end of the IR is shown for selected taxa; genes in the inverted repeat are in bold. A progressive increase in IR size is seen relative to the outgroup *Chaetosphaeridium* (three characters used in the analysis are indicated). Note that there are two different modes of increase in IR size—genes become incorporated either from the LSC region margin of the IR_A (see * on figure) or the IR_B (see ** on figure), but generally not from the SSC region.

chondrial genome support *Marchantia* as outgroup to other land plants.

Our placement of the liverworts (Figs. 1, 2) as the earliest branch conflicts with the several other studies that have placed hornworts (in this study represented by *Anthoceros*) as sister to the remaining land plants. These include studies based on nuclear rDNA sequence data (Hedderson et al., 1996), mitochondrial rDNA sequence data (Duff & Nickrent, 1999), and sequence data from *rbc*L plus small subunit rDNA from all three plant genomes (Nickrent et al., 2000). The latter multigene analyses (Nickrent et al., 2000) did not strongly support any specific basal topology for land plants. However, when third codon positions in *rbc*L were down-weighted or excluded (due to supposed saturation), then hornworts were strongly supported as the sister to the rest of land plants (Nickrent et al., 2000).

These studies conflict with our finding of a sister relationship between *Anthoceros* (our hornwort exemplar) and tracheophytes (vascular plants), supported by the expansion of the inverted repeat (IR) region by three genes (Appendix 1, character 1) and the inclusion of *rps*12 in the IR_B (Appendix 1, character 27). Because hornworts have an indeterminate sporophyte, they have often been cited as the probable sister group of tracheophytes (Smith, 1955; Stewart, 1983). Nevertheless, hornworts have been found to be the sister group to tracheophytes in only one comparative DNA sequence study (Lewis et al., 1997), using all three codon positions from *rbc*L. In addition, a study by Malek and Knoop (1998) showed a mitochondrial *nad*H dehydrogenase intron present in tracheophytes and *Anthoceros*, but missing from mosses (a finding consistent with a clade comprising *Anthoceros* and tracheophytes).

Our present study supports *Anthoceros* as the sister to tracheophytes because of a single structural rearrangement (Appendix 1, character 1) and the indel (insertion/deletion event) of a copy of *rps*12 (Appendix 1, character 27). The rearrangement comprises the inclusion in the IR region of two additional genes, *ndh*B and *rps*7 (see Fig. 3). In *Chaetosphaeridium*, *Marchantia*, and *Physcomitrella*, these three plastid genes are located in the large single copy region (LSC) adjacent to Inverted Repeat A (IR_A), the copy of the IR pictured to the right in graphic representations of the chloroplast chromosome. In *Anthoceros* and tracheophytes, copies of these genes are also located in Inverted Repeat B (IR_B) adjacent to the LSC, the copy of the IR pictured to the left in representations of the chloroplast chromosome. Although minor fluctuations in the size of the IR regions are common within angiosperms (Goulding et al., 1996; Plunkett & Downie, 2000), increases in size that comprise several genes seem to be relatively rare (Palmer, 1991; Boudreau & Turmel, 1995). When such changes do occur, they often define clades also recognized morphologically, as in Campanulaceae s.l. (Cosner et al., 1997; Knox & Palmer, 1999).

Nevertheless, as the interfaces of the IR_A and IR_B with the LSC of the plastome seem to be regions subject to size fluctuation in land plants, the possibility of convergence in this character cannot be dismissed. It has been posited that such size fluctuations in the IR region could be caused by gene conversion (Morton & Clegg, 1993; Goulding et al., 1996). Though the phenomenon (Goulding et al., 1996) has only been examined in closely related angiosperms exhibiting small differences in IR regions, the explanation could also explain larger IR size differences between more distant taxa. That such changes have happened can be inferred by comparing the number and identity of genes in the IR across land plants (Fig. 3). During the evolution of land plants, there has been a general increase in size of the IR regions as genes in the LSC bordering either IR_A or IR_B are incorporated into the IR regions. Although the trend has been generally toward an increase in size (e.g., from *Physcomitrella* to *Anthoceros* and from *Anthoceros* to *Psilotum* and *Adiantum*), some subsequent gene copy loss has occurred. Integration of genes into the IR regions from the small single copy region (SSC) is less common, but the gene *ndh*F is incorporated into the IR regions of *Oenothera*; in other sampled taxa, it is found as a single copy in the SSC adjacent to IR_B. Further sampling no doubt will increase our understanding of the changes to the IR regions during the evolution of land plants.

Of the three types of characters (rearrangements, gene indels, and intron presence), the presence/absence of intron characters was the least phylogenetically informative. There was very little variation in the number of introns within the land plant clade. Compared to *Chaetosphaeridium* with one intron in the gene *clp*P, land plants had 2 introns in this gene (Appendix 1, character 40). Similarly, *Chaetosphaeridium* and *Marchantia* had only one intron in *ycf*3, while the other taxa sampled had two introns in this gene. This latter character (Appendix 1, character 38) was the synapomorphy supporting monophyly of all land plants minus liverworts. This supports the finding of Qiu et al. (1998), who found the presence (and putative gain) of three introns in mitochondrial genes served as synapomorphies for land plants minus *Marchantia*.

Several well-defined clades are supported in this analysis. Tracheophytes are characterized by two major synapomorphies: an increase in the size of the IR region due to the incorporation of several genes from the LSC (Appendix 1, character 11) and a large gene rearrangement in a section of the LSC not far from IR_A (Appendix 1, character 9). The angiosperm clade (Fig. 2) is supported by four putative synapomorphies including the addition of *ycf*2 in the IR region (Appendix 1, character 24) and by the inferred inclusion of *rpl*23 and *rpl*2 from the IR_B end of LSC into the IR region (Appendix 1, character 10). Neither seed plants nor monilimorphs (Pryer et al., 2001) are well supported as monophyletic clades by our genomic character set. Nevertheless, further whole chloroplast genome sampling (currently underway in the Green Tree of Life project, see: ⟨http://ucjeps.berkeley.edu/TreeofLife/⟩) may reveal additional synapomorphies for these groups.

Rokas and Holland (2000) list different types of structural data and for each type the taxonomic level at which it is most phylogenetically useful. Based on previous studies (Jansen & Palmer, 1987; Doyle et al., 1994, 1995), they determined that chloroplast gene rearrangement data is most useful at the family level. In fact, the type of genomic characters used in this study can be successfully used to address phylogenetic questions at all taxonomic levels and divergence times. Of course, as these genomic changes continue to accumulate over time, a limit is reached beyond which genomic maps will not be alignable or easily comparable. Indeed, during the course of the current study, we found that some of the non-embryophyte chlorophyte plastome maps could not be easily aligned with the 18 taxa included herein.

It is clear that these genomic data potentially are useful for addressing phylogenetic questions not amenable to solution by means of DNA sequence comparison. As the rate of genome sequencing increases, gradual integration of new genomes into our initial alignment will aid interpretation, and in turn the larger number of taxa available for structural genomic analysis will increase the explanatory power of future phylogenetic studies.

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Appendix 1. Explanation of characters used in phylogenetic analysis (refer to Table 2 and Figure 2). 0 indicates absence and 1 indicates presence of stated character state.

1. Inclusion of *rps7, ndh*B, (and *trn*L-CAA) in the inverted repeat (IR) region from the large single copy (LSC) margin of IR_A, leading to gene duplication on the LSC margin of IR_B.
2. Inversion of the gene order within the IRs.
3. Loss of IR_B.
4. Large multi-gene (> 20) inversion in what corresponds to the small single copy (SSC) area in other sampled taxa.
5. Inferred loss of *Chl*N and *Chl*L genes frmo IR_A end of the small single copy area.
6. Inclusion of *ycf*2, *trn*H-GTG, and *psb*A within the inner edge of the IRs.
7. Loss of *trn*V and *rps*12 from the LSC margin of IR_B in relation to other sampled taxa.
8. Loss of *rps*7 from what is IR_B in other sampled taxa.
9. Large multi-gene (> 25) inversion from *psb*M to *ycf*2.
10. Inclusion of *rpl*23 and *rpl*2 from IR_B end of LSC into the IR region.
11. Inclusion of *trn*P-GGG, *rpl*32, and *rpl*21 into the IR region from the SSC margin of IR_B.
12. Large multi-gene (> 50) inversion in relation to other sampled taxa.
13. Inversion of *mat*K and *trn*K-UUU in relation to other sampled taxa.
14. Multi-gene (> 10) rearrangement following *trn*G-UCC.
15. Non-alignable section in regard to other taxa.
16. Multi-gene (> 20) inversion in relation to other sampled taxa.
17. Inversion of *pet*N and *psb*M.
18. Movement of multi-gene section including *psb*A, *trn*H-CAC, and *ycf*2.
19. Loss of gene section between *psb*C and *trnf*M.
20. Inversion of 6 gene section (from *trn*G-GCC to *trn*T-ACC) in relation to other free-sporing taxa sampled.
21. Presence/absence of *rps*12 gene between *rpl*36 and *rps*8.
22. Presence/absence of *rpl*22 between *rps*3 and *rps*19.
23. Presence/absence of *trn*H-GUG between *rps*19 and *rpl*2.
24. Presence/absence of *ycf*2 between *trnI*-CAU and *ycf*15 or *trn*L-CAA.
25. Presence/absence of *ycf*15 between *ycf*2 and *trn*L-CAA.
26. Presence/absence of *trn*L-CAA between *trn*1 or *ycf*2 and *ndh*B.
27. Presence/absence of *rps*12 in IR_B.
28. Presence/absence of rps15 at SSC margin of IR_B.
29. Presence/absence of *ycf*1 at SSC margin of IR_B.
30. Presence/absence of *rpl*21 between *ndh*F and *rpl*32.
31. Presence/absence of *trn*P-GGG between *rpl*32 and *trn*L-UAG.
32. Absence of *ycf*1 adjacent to *rps*15 (possible pseudogene present).
33. Presence/absence of *ndh*J between *trn*F-GAA and *ndh*K.
34. Intron missing from gene (pseudogene) of *rpl*2.
35. Intron missing from gene *rps*12.
36. Intron missing from gene *atp*F.
37. Intron missing from gene *rpo*C1.
38. Second intron missing from gene *ycf*3.
39. First intron missing from gene *clp*P.
40. Second intron missing from gene *clp*P.

MOLECULAR EVOLUTION OF THE CHLOROPLAST *TRN*L-F REGION IN LAND PLANTS[1]

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Abstract

The *trn*L group I intron and the intergenic spacer between *trn*L and *trn*F are among the most widely utilized non-coding DNA regions in plant systematics. The *trn*L-F region is probably cotranscribed and located upstream of $trnT_{UGU}$ in the large single copy region of the chloroplast genome. Whereas in algal chloroplast genomes the three tRNA genes are widely scattered, they are found in tandem in land plants. This unique arrangement, therefore, seems to be a synapomorphy for land plants. Secondary structure analysis of the group I $trnL_{UAA}$ intron shows that high sequence variability is confined to certain stem-loop regions (P6, P8), whereas elements of the catalytic core (P, Q, R, S) are highly conserved across land plants. Considerable length differences, particularly in the stem loop P8, as well as in the *trn*T-L and *trn*L-F intergenic spacers, were observed among the diverse land plant lineages. Sequences found within mosses and liverworts were generally shorter than in most other lineages. It appears that certain elements in the *trn*L-F spacer and in the most variable P8 stem-loop region of the *trn*L intron have originated by independent nucleotide additions. These elements do not share a common evolutionary history across all land plant lineages below seed plants because internally repeated sequence segments have further diversified, actually being internal paralogous stretches. Most of the length variation in the *trn*L-F spacer occurs between the putative sigma^{70}-type bacterial promoter motif upstream of *trn*F and the *trn*L 3′ exon. Apart from leptosporangiate ferns, lycophytes, and Gnetales this conserved promoter motif is present in most of the land plant lineages sampled as well as in *Chaetosphaeridium.* Structural data were used to assess the effect that compensating base pair changes (CBC) in helical elements of the intron have for phylogenetic reconstructions. Such changes significantly contribute to the phylogenetic structure of the data set. Although the inclusion of CBCs in general violates the requirement of character independence, their effect is rather comparable to weighting characters. The contribution of CBCs to the total amount of phylogenetic signal is found to be less significant within terminal land plant lineages, such as pleurocarpous mosses or angiosperms, because helical elements are conserved in their primary sequence and variation is lower within these terminal lineages.

Key words: chloroplast DNA, coevolution, group I intron, land plants, molecular evolution, promoter, *trn*L-F region.

The *trn*T-F region is located in the large single copy (LSC) region of the chloroplast genome in close proximity to *rps*4 and comprises the intergenic spacers (IGS) between the tandemly arranged tRNA genes $trnT_{UGU}$, $trnL_{UAA}$, and $trnF_{GAA}$, as well as the $trnL_{UAA}$ group I intron, which interrupts the anticodon loop of $tRNA^{Leu}$ (Fig. 1). Since the introduction of universal primers (Taberlet et al., 1991), the *trn*L intron and both spacers of the *trn*T-F region have become one of the most widely used chloroplast markers for phylogenetic analyses in plants (see Soltis & Soltis, 1998; Quandt & Stech, 2003). In particular, the *trn*L intron alone or in combination with other genomic regions has been used in numerous studies ranging from analyses of the genetic diversity in endosymbiontic *Nostoc* (e.g., Paulsrud & Lindblad, 1998; Paulsrud et al., 2000; Costa et al., 2001) to infraspecific and generic (e.g., van Ham et al., 1994; Gielly & Taberlet, 1996; Sang et al., 1997; Meißner et al., 1998; Frey et al., 1999; Wikström et al., 1999; Bakker et al., 2000; Shaw, 2000; Yang et al., 2002; Burleigh & Holtsford, 2003; Plana, 2003) as well as familial level relationships in different plant lineages (e.g., Ma-

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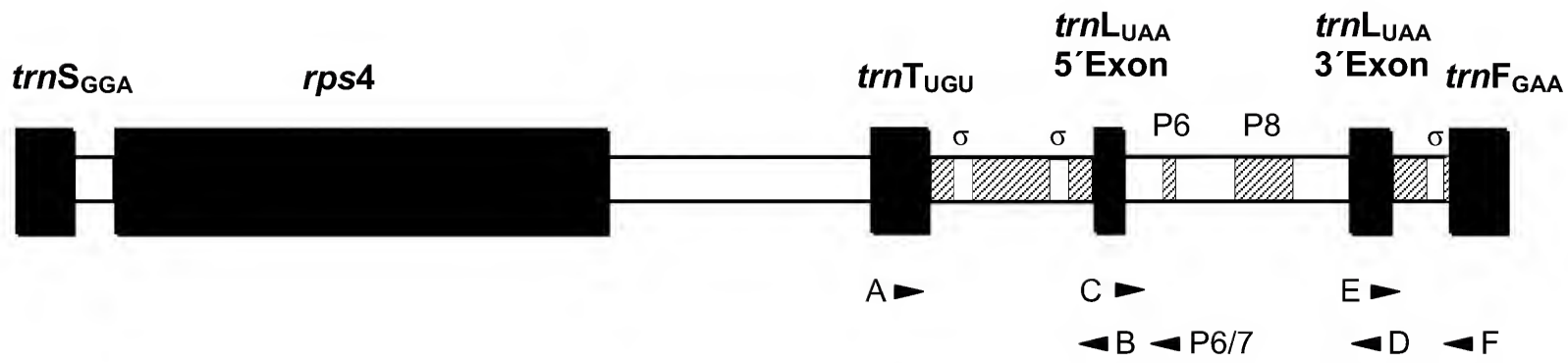


Figure 1. The *trn*T_{UGU}–*trn*F_{GAA} region in land plants. Coding regions are represented by black boxes, highly length variable regions by hatched boxes. Size and position of length variable regions change with study group. For the situation in angiosperms we refer to Borsch et al. (2003). σ indicate sites with putative promoter elements (compare Quandt & Stech, 2003). Locations of primers are indicated below, with *trn*P6/7 being designed in this study. Length of the region is presented based on the situation found in *Campylopus flexuosus*.

nen et al., 1994; Bayer & Starr, 1998; Kajita et al., 1998; Vanderpoorten et al., 2002; Hauk et al., 2003; Stech et al., 2003a). More recently, the region was employed at higher taxonomic levels with different success in the various land plant lineages (e.g., Renner, 1999; Buck et al., 2000; Stech & Frey, 2001; Borsch et al., 2003; Sauquet et al., 2003). Although high performance in angiosperms at comparatively deep nodes has been shown, culminating in the study of early diverging angiosperms (Borsch et al., 2003), the region appears to be less effective at ordinal level within mosses (see Buck et al., 2000; Shaw et al., 2003). However, the region was informative in other bryophyte lineages such as within liverworts (Wheeler, 2000; Stech & Frey, 2001) and hornworts (Stech et al., 2003a). Because it is structurally highly conserved, the intron is expected to be the most sustained part of the *trn*T-F region, whereas both spacers are more variable with respect to length and substitutions, especially the *trn*T-L spacer (Small et al., 1998; Borsch et al., 2003; Quandt & Stech, 2004). Therefore, both spacers are often used at the population level or to infer relationships among species (e.g., Böhle et al., 1996; Meißner et al., 1998; Wikström et al., 1999; Geada-Lopez et al., 2002).

Recent studies indicate that the entire *trn*T-F region can be successfully used above the family level if certain well-defined mutational hotspot segments are excluded from phylogenetic analysis. As Borsch et al. (2003) showed, the *trn*T-F region can not only be used to reconstruct deep level relationships among angiosperms, but it even provides high resolution and support similar to those derived from multi-gene, multi-genome analyses (e.g., Soltis et al., 1999; Qiu et al., 2000; Zanis et al., 2002). Thus, the question is, would the region be equally useful at even deeper levels and what would be the limitations at those levels? In spite of the diverse application of the *trn*L intron, there has been no attempt to study evolutionary patterns of the *trn*T-F region across land plants so far. Here we focus on the *trn*L intron and the *trn*L-F intergenic spacer, as *trn*L_{UAA} and *trn*F_{GAA} are thought to be cotranscribed in land plants (Kanno & Hirai, 1993).

The early evolutionary history of the *trn*L_{UAA} intron has been documented by comparing intron sequences of plastid genomes and cyanobacteria (Kuhsel et al., 1990; Besendahl et al., 2000). The results suggest that it is one of the most ancient introns found so far (Paquin et al., 1997), which entered eukaryotes through cyanobacterial endosymbiosis (Kuhsel et al., 1990; Paquin et al., 1997). In contrast to eukaryotes, where the *trn*L intron is assumed to be immobile (Kuhsel et al., 1990; Xu et al., 1990), some authors suggest a more complex evolutionary mode and history for the $tRNA^{Leu}$ introns in cyanobacteria. It is noteworthy that this might involve lateral transfer as well as independent gains and losses (Rudi & Jakobsen, 1997, 1999; for a critical discussion see Costa et al., 2002). In addition, homologous recombination among different loci has been reported from cyanobacterial *trn*L_{UAA} introns (Costa et al., 2002). In green plants, it is generally agreed that the *trn*L intron of plastids has diversified from a common ancestor. In concordance with a single origin of chloroplasts (Nelissen et al., 1995; Delwiche & Palmer, 1997), all *trn*L_{UAA} genes form a monophyletic lineage comprising only *trn*L orthologues (Kuhsel et al., 1990; Paquin et al., 1997; Rudi & Jakobsen, 1999; Besendahl et al., 2000; Costa et al., 2002). One of the characteristics of the *trn*L_{UAA} intron, and of group I introns in general, is the mosaic structure of highly conserved elements (IGS, P, Q, R, S) essential for correct splicing (Davies et al., 1982; Michel et al., 1982; Cech, 1990), alternating with less constrained regions of variable size (e.g., Cech et al., 1994; Kuhsel et al., 1990; Fig. 2). In contrast to the $tRNA^{Leu}$ group I introns of cyanobacteria that readily undergo auto-excision (Xu et al., 1990), there are so far no reports of self-

splicing activity of plastid $trnL_{UAA}$ introns, nor of plastid introns in general (Xu et al., 1990; Besendahl et al., 2000). It has, therefore, been argued that plastid group I introns depend on splicing factors, interacting with the RNA and facilitating secondary and perhaps tertiary structure formation (e.g., Akins & Lambowitz, 1987; Cech, 1988; Cech et al., 1992; Besendahl et al., 2000). The lack of any self-splicing activity in plastid introns may have led to a relaxation of selective constraints, resulting in higher evolutionary rates and, on average, greater sequence divergence of plastid introns compared to cyanobacteria (Besendahl et al., 2000).

This study aims at characterizing the *trn*L-F region, assessing its evolutionary history in land plants, and addressing its phylogenetic applicability in revealing relationships of the major land plant lineages. Attention is paid to assess homology of the different stem-loop regions of the *trn*L intron across land plants via a comparison of sequences and putative secondary structures. This is particularly important since extensive length differences have been observed (Borsch et al., 2003; Quandt & Stech, 2004), which, at least in seed plants, are confined to certain mutational hotspots (Borsch et al., 2003). We will address the question whether this high length variability in cryptogamic land plants is confined to the same hotspots as in seed plants and clarify the nature of the length mutations (also called microstructural changes) that have led to the observed extensive sequence diversity. Integrating similarity-based sequence comparisons and secondary structure analysis, we will try to unravel the evolutionary history of the region and to find out if some elements exist that do not share a common evolutionary history throughout land plants. Moreover, secondary structures allow the identification of compensatory base pair changes (CBC), which can then be tested for their effect on phylogenetic analysis.

Materials and Methods

The present analysis is based on a combination of different data sets from our previous work employing the *trn*T-F region. The angiosperm data set is largely based on the basal angiosperms data set from Borsch et al. (2003) and expanded by the addition of sequences that represent major lineages of eudicots. The bryophyte data were extracted from Quandt and Stech (2004), whereas sequence data from hornworts and ferns were obtained from Stech et al. (2003a). The additional sequences were either newly generated (16) or downloaded from GenBank, such as the two green algal sequences. Taxonomic affiliations, voucher information, and GenBank accession numbers of all included species are listed in Table 1. To amplify and sequence the *trn*T-F region, we generally used the primers reported by Taberlet et al. (1991), with the exception of primer B that was exchanged by an additional primer (trnP6/7 CAT YGA GTC TCT GCA CCT) situated in the helical elements of P6 and P7 in order to amplify and sequence the *trn*L 5′ exon. Protocols used for DNA extraction, amplification, and sequencing are given in Borsch et al. (2003), Quandt et al. (in press), and Stech et al. (2003b), respectively. The alignment was performed manually according to the rules laid out in Graham et al. (2000), Kelchner (2000), and Borsch et al. (2003) using Align (Hepperle, 2002). The alignment is available from the first author.

Two-dimensional secondary structures of the *trn*L intron (Fig. 2) were drawn according to the model proposed by Cech et al. (1994). Secondary structure calculations of the variable stem-loop regions P1 & 2, P4 & 5, P6, P8, and P9 were performed using *RNAstructure* 3.6 (Mathews et al., 2001) and the *mFOLD* server (Zuker et al., 1999). Due to the large sequence and length variation between the P8 regions across land plants, we additionally analyzed data partitions with the *ConStructV2.0* package (Lück et al., 1996, 1999), which enabled us to identify common secondary structures within multiple sequence alignments. To assess the intron variation among the different lineages, we analyzed the stem-loop regions P8 and P6 separately from the remaining intron sequences. As P6.0 is part of the conserved elements P and Q, it was excluded from the statistical analyses of P6, abbreviated thereafter as [P6]. The remaining portion of the intron included the stem-loop regions P1–5, P7, and P9, plus the conserved helix P6.0 of P6. As this portion also contained the conserved elements P, Q, R, and S (as well as P3 (E, E′) and P1–2), we chose the abbreviation [PQRS].

A maximum likelihood analysis was carried out to evaluate the phylogenetic signal in the data set. The reconstruction assumed a submodel (TIM) of the general time reversible model (GTR + G + I), with rate variation among sites following a gamma distribution (four categories represented by mean), and the molecular clock not enforced. According to the Akaike Information Criterion (AIC, Akaike, 1974) TIM + G + I was chosen as the model that best fits the data, as calculated with Modeltest 3.06 (Posada & Crandall, 2001) employing the windows front-end (Patti, 2002). Heuristic searches were run in PAUP* 4.0b10.

To test the impact of compensating base pair

Table 1. Taxa used in the study, their respective families, source of material, location of voucher specimens, and GenBank accession numbers.

Taxon	Family	Geographical origin	Voucher/herbarium location	GenBank No. (Publication)
Angiosperms				
Acorus gramineus Soland.	Acoraceae	Germany, BG Bonn	*Borsch 3458* (BONN)	AY145336 (Borsch et al., 2003)
Aextoxicon punctatum Ruiz & Pav.	Aextoxicaceae	Germany, BG Bonn	*Borsch 3459* (BONN)	AY145362 (Borsch et al., 2003)
Akebia quinata (Houtt.) Decne.	Lardizabalaceae	Germany, BG Bonn	*Borsch 3412* (BONN)	AY651844 (this study)
Amborella trichopoda Baill.	Amborellaceae	U.S.A., California, Sta. Catarina Bot. Gard.	*Borsch 3480* (BONN)	AY145324 (Borsch et al., 2003)
Annona muricata L.	Annonaceae	Germany, BG Bonn	*Borsch 3460* (BONN)	AY145352 (Borsch et al., 2003)
Arachis curziana Krapov., W. C. Greg. & C. E. Simpson	Fabaceae	Bolivia	*KSSc 36024* (IPGRI)	AY651848 (this study)
Aristolochia pistolochia L.	Aristolochiaceae	France	*Borsch 3257* (FR)	AY145341 (Borsch et al., 2003)
Austrobaileya scandens C. T. White	Austrobaileyaceae	Germany, BG Bonn	*Borsch 3464* (BONN)	AY145326 (Borsch et al., 2003)
Brassica nigra (L.) W. D. J. Koch	Brassicaceae	—	—	AF451579 (Yang et al., 2002)
Buxus sempervirens L.	Buxaceae	Germany, BG Bonn	*Borsch 3465* (BONN)	AY145357 (Borsch et al., 2003)
Cabomba caroliniana A. Gray	Cabombaceae	U.S.A., Virginia	*Ludwig s.n.* (VPI)	AY145328 (Borsch et al., 2003)
Calycanthus floridus L. var. *laevigatus* (Willd.) Torr. & A. Gray	Calycanthaceae	Germany, BG Bonn	*Borsch 3455* (BONN)	AY145349 (Borsch et al., 2003)
Ceratophyllum demersum L.	Ceratophyllaceae	U.S.A., Virginia	*Wieboldt 16073* (VPI)	AY145335 (Borsch et al., 2003)
Chloranthus brachystachys Blume	Chloranthaceae	Germany, BG Bonn	*Borsch 3467* (VPI)	AY145334 (Borsch et al., 2003)
Chusquea coronalis Soderstr. & C. E. Calderón	Poaceae	U.S.A., Missouri Bot. Gard.	MBG 896581/K	AY651842 (this study)
Dicentra eximia (Ker Gawl.) Torr.	Fumariaceae	Germany, BG Bonn	*Borsch 3468* (BONN)	AY145361 (Borsch et al., 2003)
Erodium circutarium (L.) L'Hér. ex Aiton	Geraniaceae	Germany, Ahrtal	*Borsch 3483* (BONN)	AY651847 (this study)
Houttuynia cordata Thunb.	Saururaceae	Germany, BG Bonn	*Borsch 3481* (BONN)	AY145344 (Borsch et al., 2003)
Illicium floridanum J. Ellis	Illiciaceae	U.S.A., Florida	*Borsch & Wilde 3104* (VPI, FR)	AY145325 (Borsch et al., 2003)
Lactoris fernandeziana Phil.	Lactoridaceae	(DNA from T. Stuessy)	*Stuessy s.n.*	AY145324 (Borsch et al., 2003)
Liriodendron tulipifera L.	Magnoliaceae	U.S.A., Virginia	*Slotta s.n.* (VPI)	AY145356 (Borsch et al., 2003)
Magnolia virginiana L.	Magnoliaceae	U.S.A., Maryland	*Borsch & Neinhuis 3289* (VPI)	AY145354 (Borsch et al., 2003)
Myrothamnus moschatus (Baill.) Baill.	Myrothamnaceae	Germany, BG Bonn 11396	*Fischer & Höller s.n.* (BONN)	AY651845 (this study)
Nelumbo nucifera Gaertn. subsp. *lutea* (Willd.) Borsch & Barthlott	Nelumbonaceae	U.S.A., Missouri	*Borsch & Summers 3220* (FR)	AY145359 (Borsch et al., 2003)
Nicotiana tabacum L.	Solanaceae	—	—	NC001879 (Shinozaki et al., 1986)

Table 1. Continued.

Taxon	Family	Geographical origin	Voucher/herbarium location	GenBank No. (Publication)
Nuphar lutea (L.) Sibth.	Nymphaeaceae	Germany	*Borsch 3337* (FR)	AY145330 (Borsch et al., 2003)
Nymphaea odorata Aiton subsp. *odorata*	Nymphaeaceae	U.S.A., Georgia	*Borsch & Wilde 3132* (VPI, BONN)	AY145333 (Borsch et al., 2003)
Nypa fruticans Wurmb	Arecaceae	Germany, BG Bonn	*Borsch 3463* (BONN)	AY145339 (Borsch et al., 2003)
Orontium aquaticum L.	Araceae	Germany, BG Bonn	*Borsch 3457* (BONN)	AY145338 (Borsch et al., 2003)
Phragmites australis (Cav.) Trin. ex Steud.	Poaceae	U.S.A., Virginia	*Fleming s.n.* (VPI)	AY651843 (this study)
Piper angustum Spreng.	Piperaceae	U.S.A., Missouri	Acc. 910150	AY145345 (Borsch et al., 2003)
Platanus occidentalis L.	Platanaceae	U.S.A., Virginia	*Slotta s.n.* (VPI)	AY145358 (Borsch et al., 2003)
Schisandra rubriflora (Franch.) Rehder & E. H. Wilson	Schisandraceae	Germany, BG Bonn	*Borsch 34477* (BONN)	AY145327 (Borsch et al., 2003)
Spinacia oleracea L.	Amaranthaceae	—	—	NC002202 (Schmitz-Linneweber et al., 2001)
Trochodendron aralioides Siebold & Zucc.	Trochodendraceae	Germany, BG Bonn	*Borsch 3478* (BONN)	AY145360 (Borsch et al., 2003)
Utricularia vulgaris L.	Lentibulariaceae	—	—-	AF482681 (Jobson & Albert, 2002)
Vitis riparia Michx.	Vitaceae	Germany, BG Bonn	*Borsch 3598* (BONN)	AY651846 (this study)
Gymnosperms				
Araucaria araucana (Molina) C. Koch	Araucariaceae	Germany, BG Bonn	*Borsch 3462* (BONN)	AY145321 (this study)
Cycas siamensis Miq.	Cycadaceae	Germany, BG Bonn	*Quandt E10038* (BONN)	AY651841 (this study)
Ginkgo biloba L.	Ginkgoaceae	U.S.A., Virginia Tech Bot. Gard.	*Borsch 3469* (VPI)	AY145323 (Borsch et al., 2003)
Gnetum gnemon L.	Gnetaceae	Germany, BG Bonn	*Borsch 3470* (BONN)	AY304546 (Borsch et al., 2003)
Pinus thunbergiana (Parl.) Franco	Pinaceae	—	—	NC001631 (Wakasugi et al., 1994)
Leptosporangiate ferns				
Adiantum capillus-veneris L.	Pteridaceae	—	—	NC004766 (Wolf et al., 2003)
Cyathea pectinata Ching & S. H. Wu	Cyatheaceae	—	—	AJ505930 (Wang, unpublished)
Dicksonia antarctica Labill.	Dicksoniaceae	Germany, BG Berlin	*s.n.* (B)	AY327843 (Stech et al., 2003a)
Gleichenia dicarpa R. Br.	Gleicheniaceae	Germany, BG Bonn	*Borsch 3694* (BONN)	AY651838 (this study)
Osmunda regalis L.	Osmundaceae	Germany, Bonn	*Quandt E10018* (BONN)	AY651837 (this study)
Polypodium vulgare L.	Polypodiaceae	Germany, BG Bonn	*Quandt E10040* (BONN)	AY651840 (this study)
Salvinia oblongifolia Mart.	Salviniaceae	Germany, BG Bonn	*Quandt E10039* (BONN)	AY651839 (this study)
Eusporangiate ferns				
Angiopteris madagascariensis de Vriese	Marattiaceae	Germany, BG Bonn	*Quandt E10041* (BONN)	AY651836 (this study)

Table 1. Continued.

Taxon	Family	Geographical origin	Voucher/herbarium location	GenBank No. (Publication)
Botrychium lunaria (L.) Sw.	Ophioglossaceae	Germany, BG Berlin	*Hamman & Scholz s.n.* (BSB)	AY327841 (Stech et al, 2003a)
Ophioglossum petiolatum Hook.	Ophioglossaceae	Germany, BG Berlin	*s.n.* (B)	AF071849/AY138447 (Stech et al., 2003a)
Whisk ferns				
Psilotum nudum (L.) P. Beauv.	Psilotaceae	Germany, BG Berlin	*s.n.* (B)	AY327836 (Stech et al., 2003a)
Horse tails				
Equisetum hyemale L.	Equisetaceae	Germany, BG Bonn	*Borsch 3403* (BONN)	AY327837 (Stech et al., 2003a)
Equisetum scirpoides Michx.	Equisetaceae	—	—	AF182359 (Besendahl et al., 2000)
Clubmosses and allies				
Huperzia squarrosa (G. Forst.) Trevis.	Lycopodiaceae	—	—	AJ224557 (Wikstrom, unpublished)
Lycopodium alpinum L.	Lycopodiaceae	—	—	AJ224599 (Wikstrom, unpublished)
Selaginella tamariscina (P. Beauv.) Spring	Selaginellaceae	Germany, BG Bonn	*Quandt E10042* (BONN)	AY651835 (this study)
Hornworts				
Anthoceros punctatus L.	Anthocerotaceae	Germany, BG Cologne	*Quandt E 10027* (hb. Quandt)	AY327827 (Stech et al., 2003a)
Notothylas orbicularis (Schwein.) Sull.	Anthocerotaceae	Germany	*Frahm 5827* (hb. Frahm)	AY327828 (Stech et al., 2003a)
Mosses				
Andreaea rupestris Hedw.	Andreaeaceae	Finland	*Stech B970827.3* (hb. Stech)	AY050278/AY050285 (Stech et al., 2003a)
Bartramia stricta Brid.	Bartramiaceae	Turkey	*Quandt T3-2* (hb. Quandt)	AY651834 (this study)
Campylopus flexuosus (Hedw.) Brid.	Dicranaceae	Germany	*Stech B960905.2* (BONN)	AF129593/AF129566 (Stech et al., 2003a)
Cryphaeophilum molle (Dusén) M. Fleisch.	Cryphaeaceae	Chile	*Blöcher 42* (hb. Blöcher)	AF509544 (Quandt et al., in press)
Lembophyllum divulsum (Hook. f. & Wilson) Lindb.	Lembophyllaceae	New Zealand	*Frahm 8-25* (CHR)	AF187249 (Quandt et al., 2000)
Papillaria leuconeura (Müll. Hal.) A. Jaeger	Meteoriaceae	New Zealand	*Rey 94-176* (hb. Frey)	AF187244 (Quandt et al., 2000)
Polytrichadelphus magellanicus (Hedw.) Mitt.	Polytrichaceae	New Zealand	*Frey 98-Mo 32* (hb. Frey)	AF187237/AY051372 (Stech et al., 2003)
Pyrrhobryum latifolium (Bosch & Sande Lac.) Mitt.	Rhizogoniaceae	Taiwan	*Ahonen et al. s.n.* (H)	AY044077 (Quandt & Huttunen, 2004)
Sphagnum fallax H. Klinggr.	Sphagnaceae	—	*Capesius sterile culture* (BONN)	AY327835 (Stech et al., 2003a)

Table 1. Continued.

Taxon	Family	Geographical origin	Voucher/herbarium location	GenBank No. (Publication)
Takakia lepidozioides S. Hatt. & Inoue	Takakiaceae	Canada	*Schofield 113636A* (BONN)	AY651832 (Quandt & Stech, 2004)
Tortula atrovirens (Sm.) Lindb.	Pottiaceae	Spain	*MUB 11352* (MUB)	AY651833 (this study)
Liverworts				
Blasia pusilla L.	Blasiaceae	Austria	*Frahm s.n.* (hb. Frahm)	AY007619 (Stech & Frey, 2001)
Marchantia polymorpha L.	Marchantiaceae	—	—	NC001319 (Ohyama et al., 1986)
Metzgeria furcata (L.) Corda	Metzgeriaceae	Germany	*Frey 2-882* (hb. Frey)	AY007626 (Stech & Frey, 2001)
Monoclea forsteri Hook.	Monocleaceae	New Zealand	*Frey 94-106* (hb. Frey)	AF071837/AF081211 (Meißner et al., 1998)
Plagiochila stephensoniana Mitt.	Plagiochilaceae	New Zealand	*Pfeiffer 98-Z 96B* (hb. Frey)	AY007632 (Stech & Frey, 2001)
Symphyogyna hymenophyllum Mont. & Nees	Pallaviciniaceae	Chile	*Frey 2-871* (hb. Frey)	AF173331 (Meißner et al., 1998)
Green algae				
Chaetosphaeridium globosum (Nordst.) Kleb.	Chaetosphaeridiaceae	—	—	AF494278 (Turmel et al., 2002)
Chlorella vulgaris Beij.	Chlorellaceae	—	—	NC001865 (Wakasugi et al., 1997)

changes (CBC) on phylogenetic reconstruction, we assessed the impact of removing information gained from one half of the corresponding sites on phylogenetic structure. The term phylogenetic structure (R) is used here to denote the robustness of a tree topology as inferred from estimating the average bootstrap support per node (see also Källersjö et al., 1999). The exact calculation of R has been previously described in Quandt et al. (2003). The significance of the difference between the inclusion of (a) all CBC regions and (b) only one half out of two corresponding halves of the regions was estimated using jackknifing. Each one out of n (here: 500) jackknife replicates j comprised a pair of analyses, corresponding to (a) and (b) above. For analysis (a), $1 - (1/e)$ characters were randomly sampled, then all not previously sampled sites from the half under scrutiny were added, whereas in (b), all sites from one half were excluded. R was calculated for (a) and (b), and d defined as $R(a) - R(b)$. The standard error for d then was estimated as

$$\sigma_d = \sqrt{\frac{1}{n-1}\sum_j \left[\left(\frac{1}{n}\sum_j d_j\right) - d_j\right]^2} \qquad (1)$$

Results

Phylogenetic reconstructions in land plants using *trn*L-F

As we will argue in the discussion, the *trn*L-*trn*F arrangement seems to be a synapomorphy for land plants. Therefore, considering that an algal outgroup is needed, it leaves only the *trn*L intron for phylogenetic inferences across all land plants. A *trn*L land plant data set is further reduced due to the need of excluding the stem-loop region P8, as it is not alignable across land plants and might not even be homologous between all lineages (see discussion). Hence, any phylogenetic analysis using the *trn*L-F region across land plants is more or less limited to the conserved parts of the intron (as, e.g., in Besendahl et al., 2000). For the present analysis, the stem-loop region P8 was excluded apart from P8.0 and P8.1 (based on the secondary structure representation of *Chaetosphaeridium globosum*; Fig. 2), whereas P6 was completely retained. The resulting data matrix comprised 821 characters, of which 496 characters were constant and 305 variable, with 220 being parsimony-informative. The maximum likelihood analysis inferred a single tree (Lscore 7346.10926; length = 1445; CI = 0.382; RI = 0.661; RC = 0.252; parameter estimates: BaseFreq = (0.3951, 0.1219, 0.1896); NST = 6; Rmatrix = (1.0000, 3.3359, 0.4410, 0.4410, 4.5901); Rates = gamma; Shape = 0.8047; Pinvar

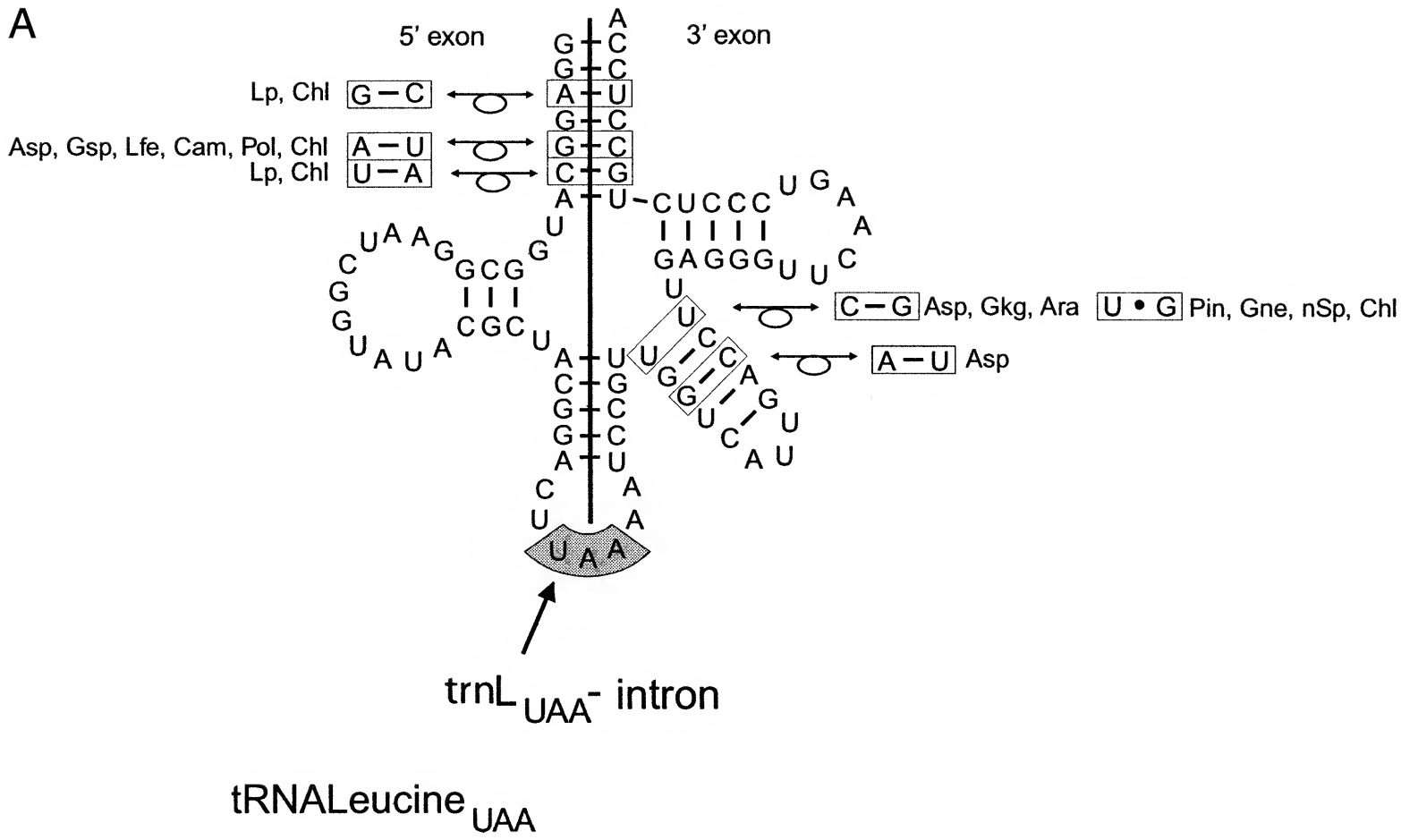


Figure 2. Secondary structure representations. —A. $tRNA^{Leu}$ encoded by the *trn*L_{UAA} gene of *Chaetosphaeridium globosum*. The position of the *trn*L_{UAA} intron in the gene is indicated. —B. *trn*L_{UAA} intron of *Chaetosphaeridium globosum*. P1–P9 annotates the different helical elements. The conserved sequence motifs IGS, P, Q, R (i.e., R_1 + R_2), S, and the G preceding the *trn*F_{GAA} gene are depicted in gray boxes. Examples of their occurrence in the different taxa or lineages are given aside, which represent a consensus, as exceptions might occur within and between lineages. Adi = *Adiantum*, Ara = *Araucaria*, Asp = angiosperms, Bry = Bryopsida, Cam = *Campylopus*, Chl = *Chlorella*, Equ = *Equisetum*, Gkg = *Ginkgo*, Gne = *Gnetum*, Gsp = gymnosperms, Hws = hornworts, Ju = Jungermanniopsida, Lfe = leptosporangiate ferns, Lp = land plants, Lws = liverworts, Lyc = lycophytes, Ma = Marchantiopsida, nSp = non-seedplants, Nym = Nymphaeaceae s.l. sensu Borsch et al. (2003), Oph = Ophioglossidae, Op = *Ophioglossum*, Pin = *Pinus*, Ppd = *Polypodium*, Pol = *Polytrichadelphus*, Sph = *Sphagnum*.

= 0.1272) shown in Figure 3 that is largely congruent in topology with the multi-gene analysis performed by Pryer et al. (2001), and illustrates that some reliable signal is present in the matrix.

SEQUENCE DIVERSITY IN THE *TRN*L INTRON AND THE *TRN*L-F SPACER

Throughout land plants the length and GC (G = Guanine, C = Cytosine) content of the *trn*L intron and the *trn*L-F spacer appear to be highly variable (Table 2), with the *trn*L intron being overall more conserved than the *trn*L-F spacer. Within the group I intron the stem-loop region P8 accounts for most of the length variability (Fig. 4). Land plant lineages with a position in the tree above bryophytes (excluding hornworts) tend to have longer P8 stem-loop regions, although there are exceptions as will be discussed below. The *trn*L-F spacer exhibits a much clearer trend towards increased length above bryophytes (excluding hornworts), with noteworthy exceptions such as in the Gnetales where sequences are not only short but also highly divergent at the nucleotide level and in *Orontium* (Araceae), which has a big deletion (Fig. 5). The GC content, however, is distinctly higher in the conserved elements of the intron ([PQRS]) compared to the adjacent stem-lop regions [P6] and P8 (Fig. 6). Consensus sequences of the conserved elements P, Q, R, and S are provided in Table 3. As a consequence, variation in the overall GC content seems to be influenced by the proportion of nucleotides in conserved elements to nucleotides in variable stem-loop elements.

SECONDARY STRUCTURES AND COMPENSATORY BASED PAIR CHANGES

In this study we have calculated secondary structures for each stem-loop element (P1 plus P2, P4 plus P5, P6, P8, and P9) for representatives of all major land plant lineages that are represented in Figure 2 by the *trn*L intron secondary structure found in *Chaetosphaeridium globosum*. Examples of compensatory base pair changes are summarized according to the secondary structure representation derived for *Chaetosphaeridium globosum*. Also, different taxa of the same lineage were analyzed when

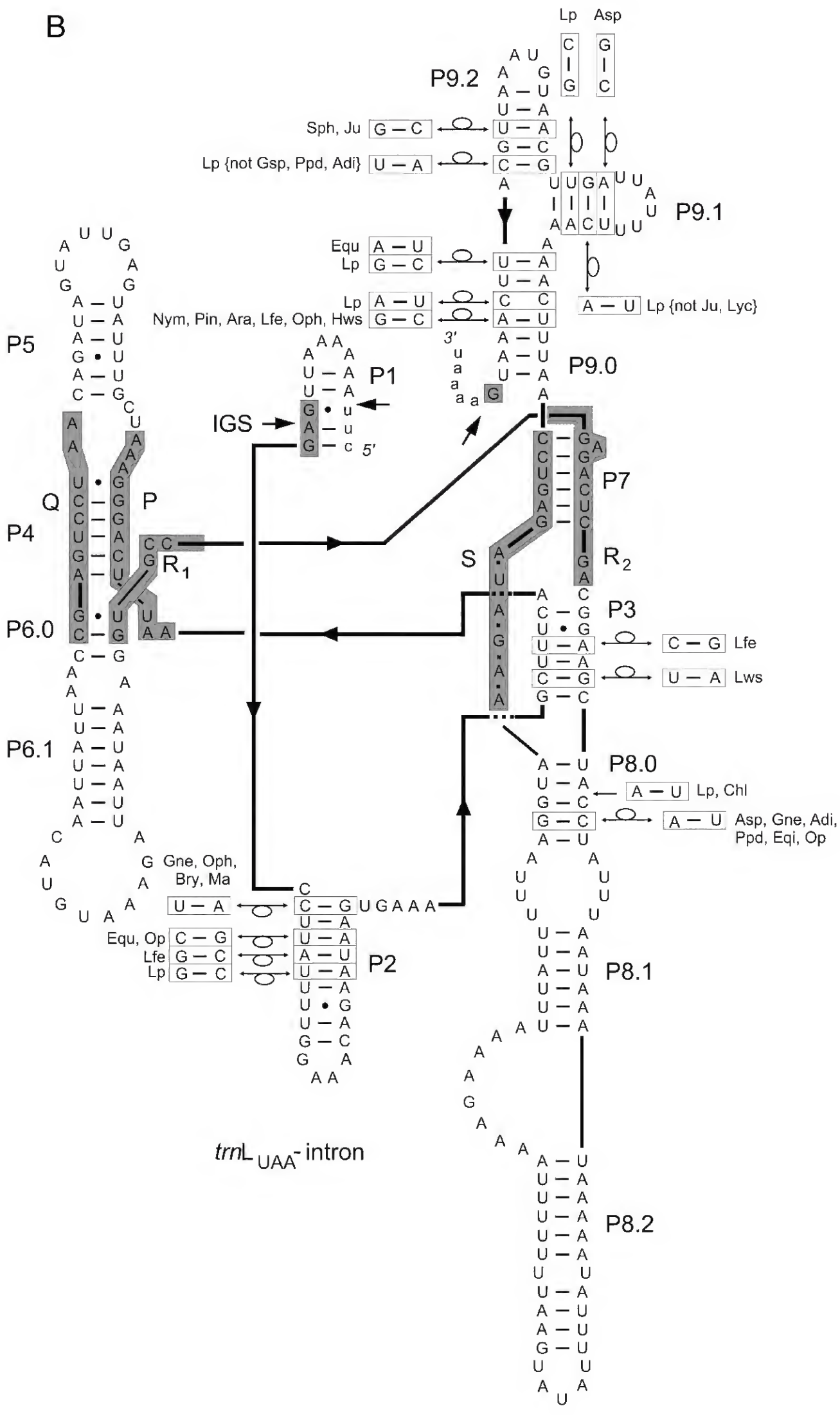


Figure 2. Continued.

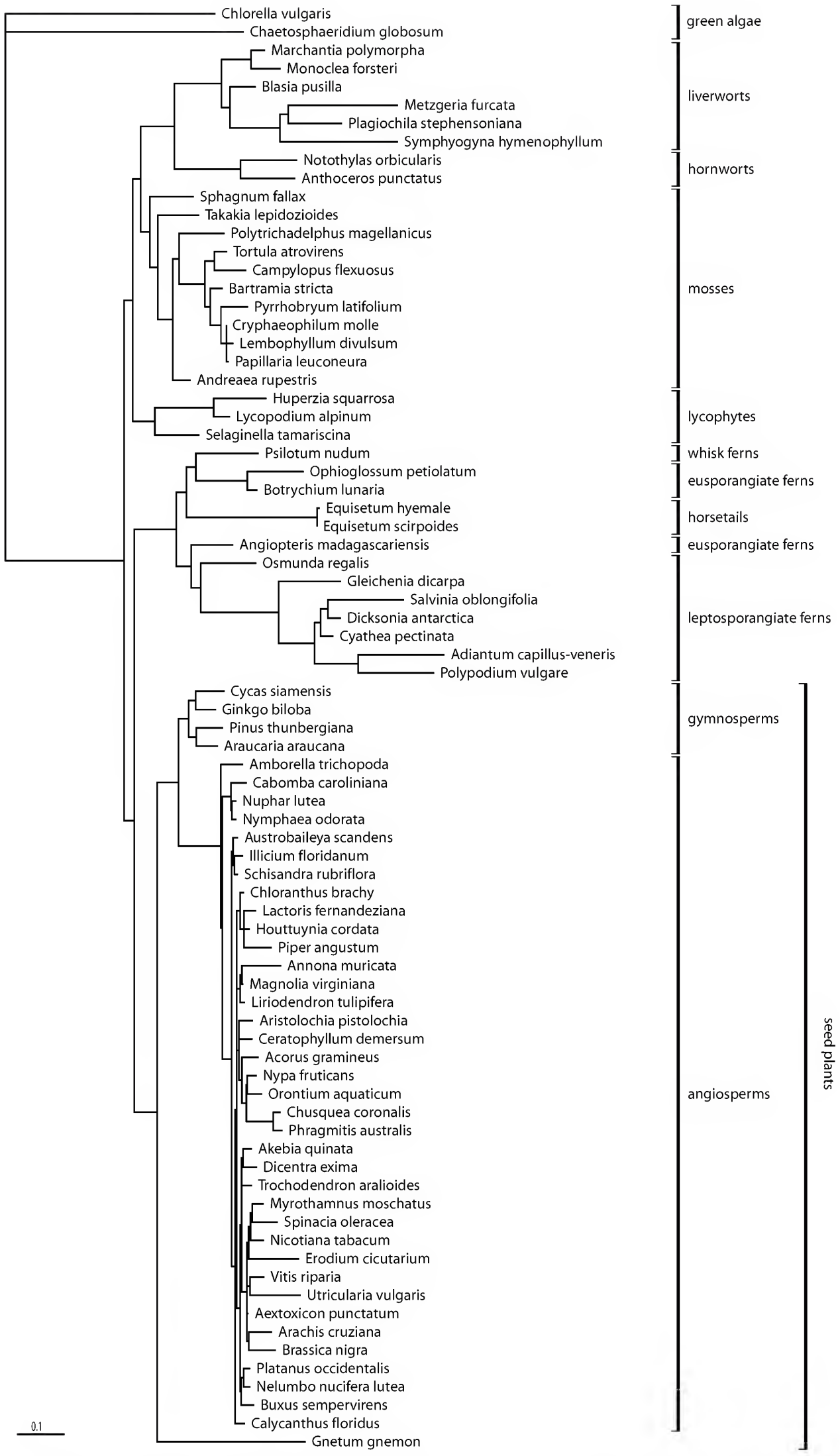


Figure 3. Maximum likelihood (lscore 7352.57593; length 1452, CI = 0.380, RI = 0.655, RC = 0.249) tree based on the *trn*L intron, excluding P8 apart from P8.1. This single gene tree does not necessarily represent the authors' opinion of the relationships among the different land plant lineages. Low number of characters could exaggerate the effect of homoplasy, resulting in various lineage misplacements, such as *Aristolochia pistolochia,* and also result in short branches and reduced resolution as in angiosperms. The phylogram was merely chosen to illustrate the phylogenetic power obtained from the region. The phylogram was drawn with Treegraph (Müller & Müller, in press).

Table 2. Length [bp] and GC content [%] variation in the *trn*L-F region (mean, s.d.). PQRS = intron without the stem-loop regions P6 and P8. P6 = stem-loop region P6 without P6a. See text for explanation.

	Length	GC content
*trn*L intron	218–660 (443.7, s.d. 188.8)	22.9–45.3 (33.9, s.d. 5.2)
PQRS	157–183 (172.1, s.d. 0.7)	29.7–52.4 (40.5, s.d. 5.1)
P6	17–109 (45.7, s.d. 25.5)	0–50 (27.7, s.d. 4.5)
P8	26–360 (213.9, s.d. 149.2)	3.6–43.7 (28.7, s.d. 20.6)
*trn*L-F spacer	51–466 (288.3, s.d. 209.3)	10.5–44.0 (31.0, s.d. 13.2)

sequence length or primary base composition were conspicuously different. Characteristic P8 stem-loop structures of major land plant lineages are illustrated in Figure 7. Variation in P8 among first branching angiosperms is used as a model to illustrate the mechanisms by which the increase of sequence length in stem-loop regions can be explained (Fig. 8; see discussion), whereas differences for major lineages in the structure of P4 and P5 are contrasted in Figure 9. Assessing the impact of compensatory base pair changes on phylogenetic reconstructions, we could show that the CBCs significantly contribute to the phylogenetic structure of this land plant data set (Table 4).

Discussion

The *trn*L_{UAA} gene and its group I intron

The *trn*L_{UAA} intron shows a length spectrum ranging from 218 (*Cryphaeophilum molle* and *Equisetum* spp.) to 660 bp (*Nothothylas orbicularis*) across the sampled land plant taxa (Table 2, Fig. 4). Comparing the *trn*L_{UAA} intron of mosses (mean length 323 bp) and liverworts (mean length 321 bp) with tracheophytes (mean length 480 bp) and hornworts (577 or 660 bp), length differences are considerable. Generally, sequences found within mosses and liverworts were shorter than in most other lineages, although some groups, such as *Sphagnum,* might possess quite long *trn*L_{UAA} intron sequences (compare Quandt & Stech, 2004; Shaw, 2000). On the other hand, within tracheophytes some exceptionally short sequences were observed, such as in *Equisetum* spp., *Ophioglossum petiolatum,* or among angiosperms (e.g., *Spinacia oleracea* or *Brassica nigra*). This size variation is attributed mainly to length mutations occurring within definite elements of the intron's secondary structure (Table 2, Fig. 2). Whereas in [PQRS] almost no length variation occurs among the different land plant lineages and even the algal outgroups (Fig. 4), two stem-loop regions, [P6] and P8, account for almost the complete length variation. Length differences are so extensive that alone the P8 region of angiosperms is comparable to the total *trn*L intron in mosses. While P8 contributes more than 50% of the total intron size in angiosperms, length variation of [P6] is very limited. Nevertheless, similar trends as in P8 could be observed. [P6] tends to be longer in seed plants and leptosporangiate ferns than in the other land plant lineages, except for Anthocerotophyta (Fig. 4) with a [P6] region two to five times the length of that of any other land plant lineage (compare Stech et al., 2003a).

In addition to its length variation, the GC content of the stem-loop region P8 and [P6] differed distinctly from [PQRS] (Table 2). In each single species the GC content of P8 is lower than that of [PQRS] (Fig. 6). The mean values for the GC content across the data set indicate that [PQRS] is rather GC-rich, whereas both stem-loop regions tend to be less GC-rich. In addition, there is a positive correlation between the length of the stem-loop region in P8 and its GC content across land plants. Pearson's coefficient of correlation (r) test shows the GC content and length of P8 to be significantly correlated ($r = 0.582$; $P < 0.05$).

Sequence comparisons of the stem-loop region P8 reveal that within certain groups of taxa, such as, e.g., angiosperms or mosses, similar nucleotide sequences are observed that might be used to define a specific group. However, sequence divergence between these groups is then so high that an alignment is not possible. Using the *ConStructV2.0* package (Lück et al., 1996; Lück et al., 1999), we were not able to generate a common secondary structure of P8 for the present data set of land plants (Fig. 7). Surprisingly, even within liverworts or mosses a common structure could not be inferred (Quandt, 2002; Quandt et al., 2004). Moreover, all potentially ancestral green algal and cyanobacterial *trn*L_{UAA} introns are considerably shorter than those of most land plants, especially in P8. Thus, elongation processes must have occurred during the evolution of land plants, probably independently in different lineages. In some cases they were followed by secondary losses of large parts, such as in *Spinacia oleracea* or *Brassica nigra.*

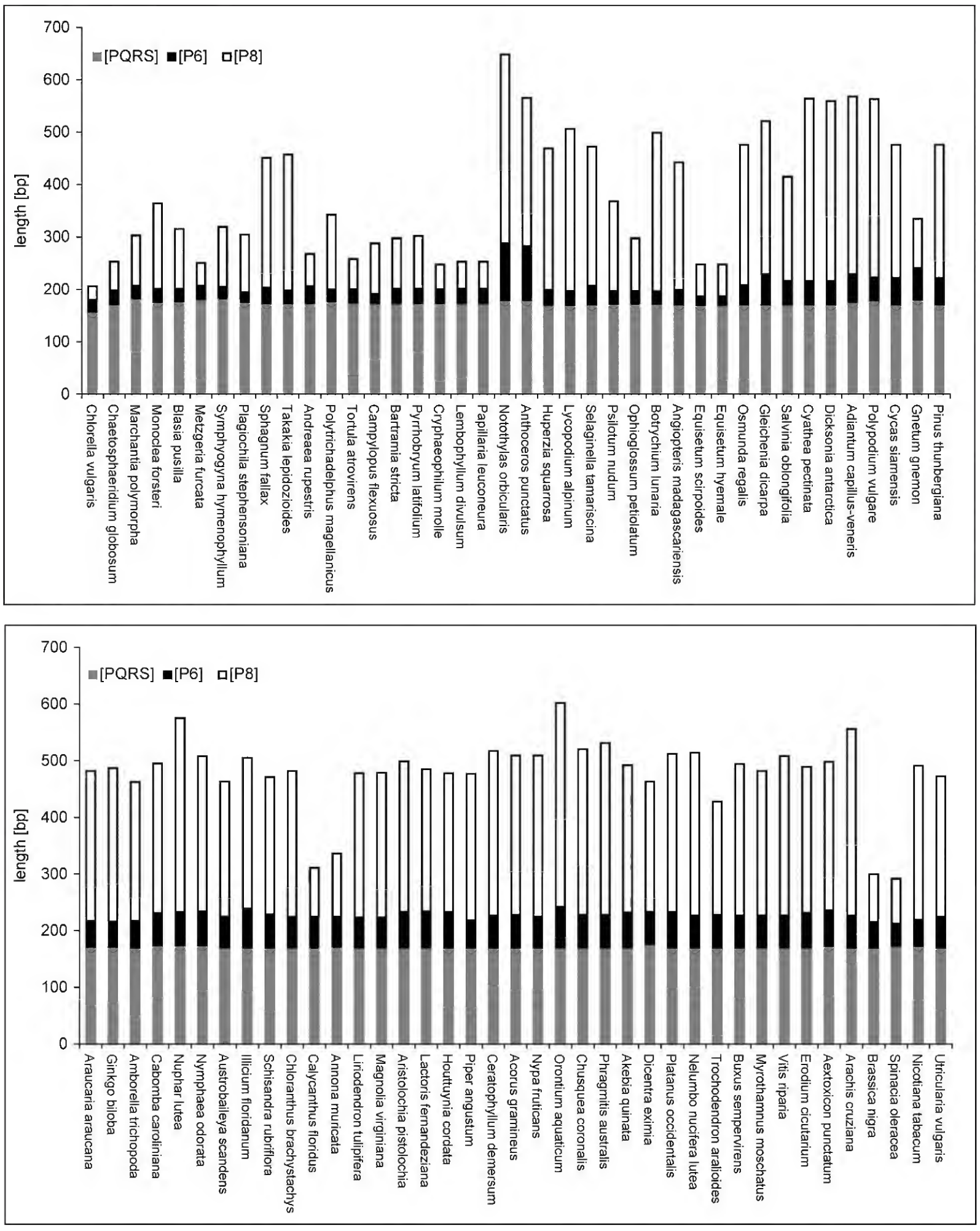


Figure 4. Length variation of the *trn*L intron in land plants and two green algal representatives. Contributions to the total length of the intron in the different species from the more conserved parts of the intron (PQRS), and both length variable stem-loop regions (P6 and P8) are indicated. PQRS = intron without the stem-loop regions P6 and P8. P6 = stem-loop region P6 without P6a. Species are arranged in systematical order.

Early diverging angiosperm lineages are well suited for providing insights into the mechanisms of P8 sequence evolution, in particular since angiosperms are undoubtedly monophyletic and the branching order of the basalmost lineages (Amborellaceae, Nymphaeales, and Austrobaileyales, respectively) has been established (Qiu & Palmer, 1999; Qiu et al., 1999, 2000; Mathews & Dono-

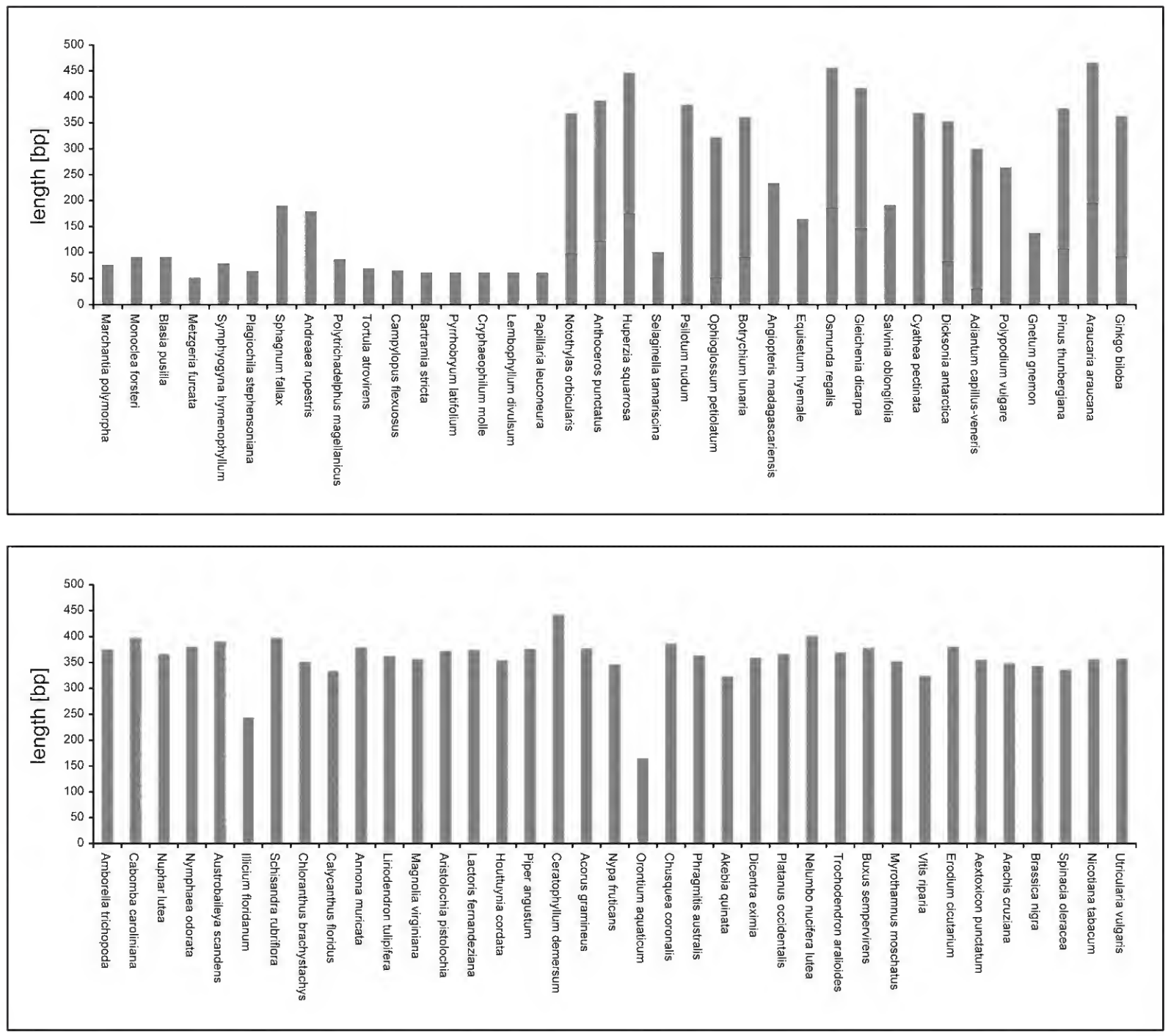


Figure 5. Length variation of the *trn*L-F spacer in the sampled land plants. Species are arranged in systematical order.

ghue, 2000; Soltis et al., 2000; Zanis et al., 2002; Borsch et al., 2003; Hilu et al., 2003). The stem-loop region P8 in *Nuphar,* which is part of the Nymphaeaceae clade, is considerably longer (up to 100 bp) than in other basal angiosperms (Borsch et al., 2003) due to AT-rich (A = Adenine, T = Thymine) repetitive elements inserted in a highly variable part of P8. A mechanistic explanation for these insertions is slipped strand mispairing (SSM), which can easily be inferred from the primary sequence. The sequence is satellite-like and exclusively consists of short AT-motifs that are prone to cause slippage (Levinson & Gutman, 1987). Plotting P8 stem-loop sizes on the basal angiosperm phylogeny (Fig. 8) further reveals that the most likely explanation for the large AT-rich P8 stem loop in *Nuphar* is an insertion process. The alternative explanation of sequence losses in the remaining basal angiosperm taxa except *Nuphar* is less parsimonious, since it would require parallel deletions in *Amborella, Cabomba, Nymphaea,* and *Illicium.* As high rates of microstructural changes are generally characteristic of satellite-like segments (e.g., Valdes et al., 1993), a rapid sequence elongation by repeated SSM events seems to be plausible. It is, therefore, feasible that a long insertion made up of repeat elements can evolve rather rapidly in a terminal lineage. Considering the topology of the land plant tree, we conclude that these insertion events must have occurred in *Nuphar* or its immediate ancestor after the evolution of the intron's P8 core structure in angiosperms. It is likely that independent SSM events occurred at different times in the other land plant lineages, resulting in various elongation events. This example may, therefore, also serve to explain the deviating sequence blocks in different lineages, such as liverworts or gymnosperms. For example, AT-dominated repeat motifs are present

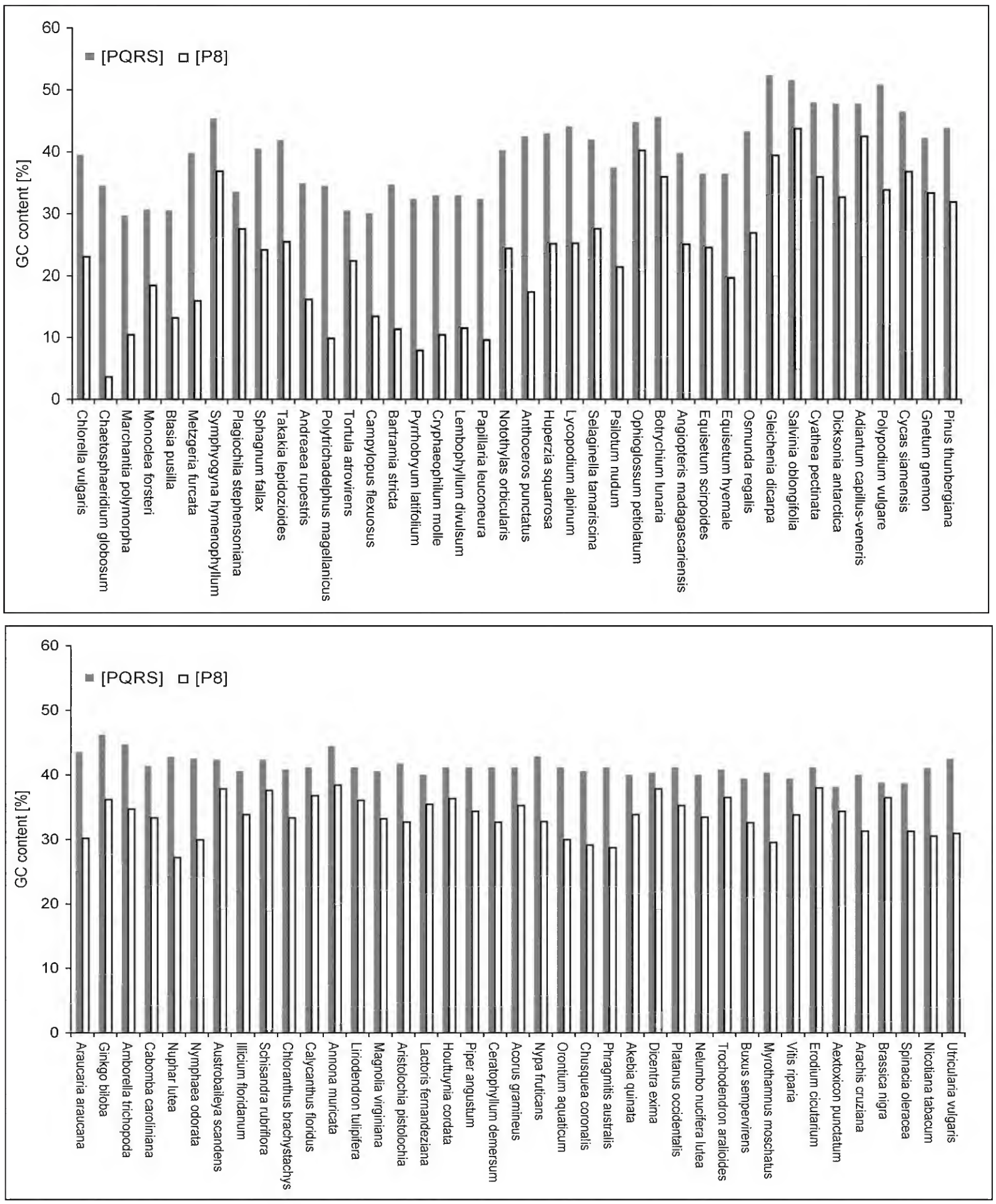


Figure 6. GC content variation of the more conserved part of the intron (PQRS) versus the highly length variable stem-loop region P8 in land plants and two green algal representatives. PQRS = intron without the stem-loop regions [P6] and P8. [P6] = stem-loop region P6 without P6.0. Species are arranged in systematical order.

in some basal pleurocarpous mosses, whereas there is a trend towards shorter sequences of P8 in the more derived pleurocarpous mosses (Quandt, 2002; Quandt & Stech, in press). Subsequently, substitutional modifications can result in an increase in GC content over time. In the case of *Nuphar*, time was not sufficient to accumulate a high number of substitutions, although few As and Ts have been replaced by Cs or Gs in the respective insertion among different species (Borsch, Müller, Hilu, Worberg, Löhne, unpublished data).

The observed correlation between the size of the P8 stem-loop region and its GC content also applies in the scenario described for *Nuphar*. It seems like-

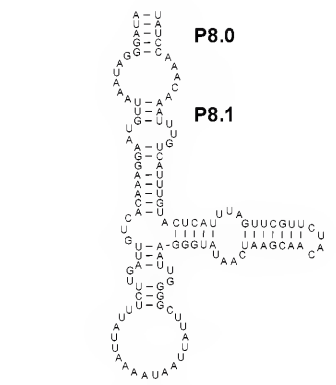
Table 3. Consensus sequences of the conserved sequence elements P, Q, R, and S (Cech, 1988) in the sampled taxa. Dots represent identical nucleotides to *Chlorella*. Differences to *Chlorella* are stated as letters according to the standard code. Lower case letters indicate a single mutation occurring in only one of the sampled species within each lineage. Numbers in parentheses indicate the number of species sampled. An asterisk indicates a missing nucleotide. The last nucleotide of P given in parentheses refers to the consensus sequence definition of Kuhsel et al. (1990).

	P	Q	R	S
Chlorella	AATTCAGGGAA*A(C)	AATCCTGAGT	GTGCAGAGACTCGA	AAGAGAGAGTCC
Chaetosphaeridium	..........*.(*)	C	.....	....T.....
liverworts (6)	wat...rg...*.(Y)	....Yt...C	..........R.	....K.....
mosses (11)	gA.........*t(C)	R........C	..........R.	....T.....
hornworts (2)	G.......W..*R(C)	.....C...C	...T........A.	....T.....
clubmosses and allies (3)	g....a.....*.(C)	R........C	..........R.	...aT.....
whisk ferns (1)	..........*.(C)	........AC	..........A.	....G.....
eusporangiate ferns (3)	a.........*.(C)	C	.....	...aT.....
horsetails (2)	..........*.(C)	C	..........A.	....T.....
leptosporangiate ferns (7)	R........R.*a(C)	C	..R.........g.	a..aT....t..
gymnosperms (5)	...c.......*c(C)	........gC	.t.........R.	...aT.g.....
angiosperms (37)	..t....a.a.AC(C)	C	..g.........R.	...aK.....

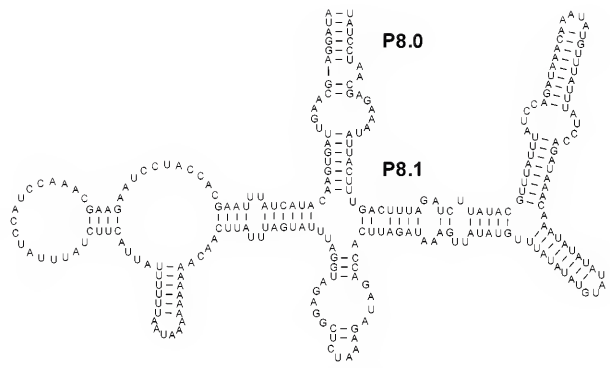
ly that additional repeats are inserted through time, during which also point mutations occur. Considering that there are certain probabilities for substituting an A or T with a G or C, it is very likely that the GC content of AT-rich stretches rises as part of a stochastic process. The question remains if there is a trigger leading to structural stabilization of elongated sequences in a given lineage. This could be the case because P8 might need to be structurally stabilized either to prevent a negative interference during the splicing process or to assist in the splicing process, possibly via interactions with splicing factors. In addition, an increase in length of P8 might hamper the formation of the catalytic core or destabilize it. Therefore, stabilization of the stem-loop region P8 via stronger GC bonds might be favorable and thus be selected for. As mentioned above, there are no reports of self-splicing activity of plastid introns (Xu et al., 1990; Besendahl et al., 2000). Correct splicing most likely depends on correct secondary and tertiary structure formation, perhaps facilitated by splicing factors and their interaction with some parts of the intron (see Akins & Lambowitz, 1987; Cech, 1988; Cech et al., 1992; Besendahl et al., 2000). Since the main difference between cyanobacterial and plastid $trnL_{UAA}$ introns centers on the length and the resulting structural differences of P8, this might be the reason for the observed lack of splicing activity in plastid $trnL_{UAA}$ introns. An interaction of the plastid P8 with some sort of splicing factor would therefore be logical. But as the elongation process presumably took place several times independently in the various land plant lineages, the splicing factors as well as their interaction with the intron might vary in different lineages.

How the conspicuously short P8 stem loop in Gnetales can be explained remains unclear. Won and Renner (submitted) report high levels of variability in substitutions and length mutations among the three genera of Gnetales (*Gnetum, Welwitschia, Ephedra*). Because promoter elements are lacking, Won and Renner (submitted) suggest that transcription of *trn*L-F and processing of the tRNA works differently in Gnetales, utilizing hairpin structures. This could also have an effect on splicing factors and lead to decreased functional constraints on the *trn*L intron. An alternative explanation would be linked to a first branching position of the Gnetales in seed plants (see below), so that a short P8 can be interpreted as a plesiomorphic condition.

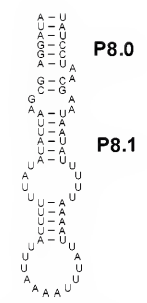
In contrast to the large length differences observed in [P6] and P8, the remaining stem-loop regions, such as P1–2, P5, and P9, were rather conserved in length and structure. Nevertheless, these



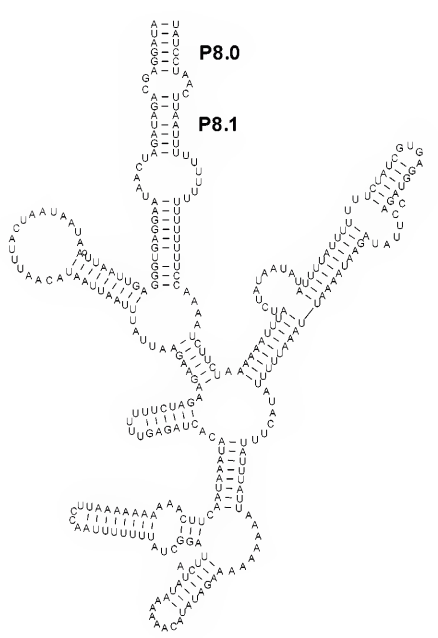
Plagiochila stephensoniana



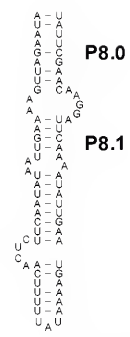
Sphagnum fallax



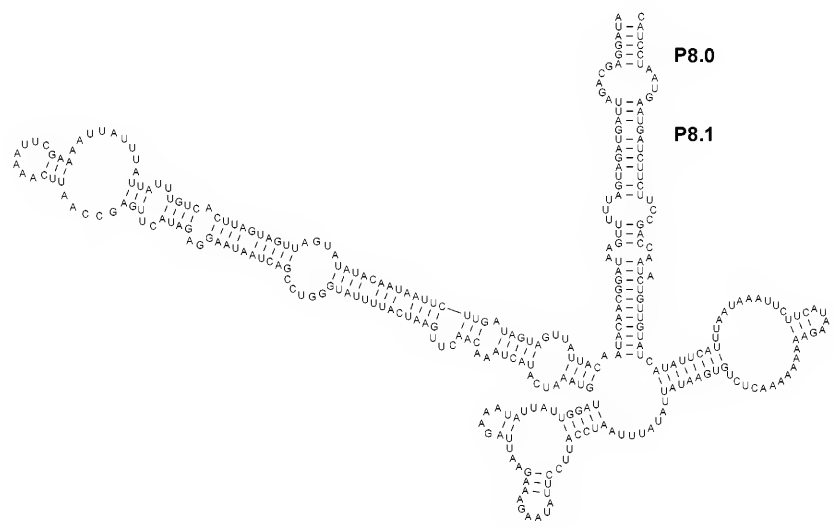
Papillaria leuconeura



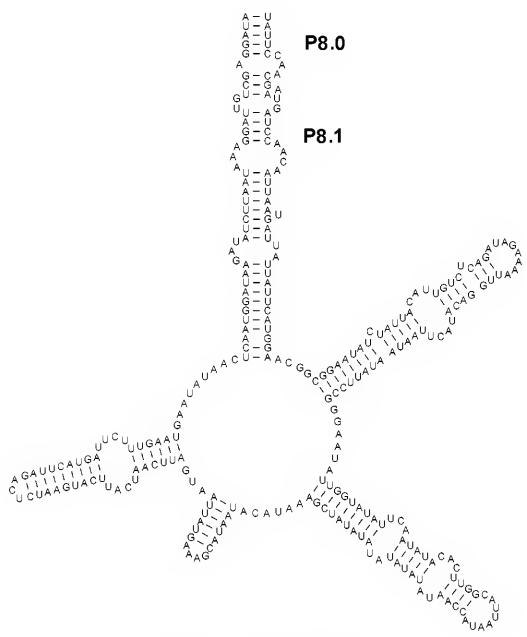
Anthoceros punctatus



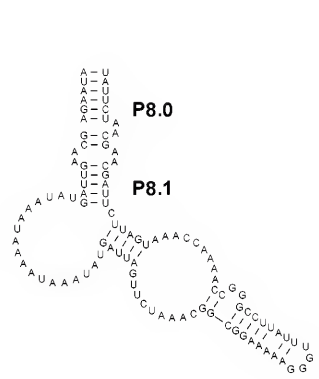
Equisetum hyemale



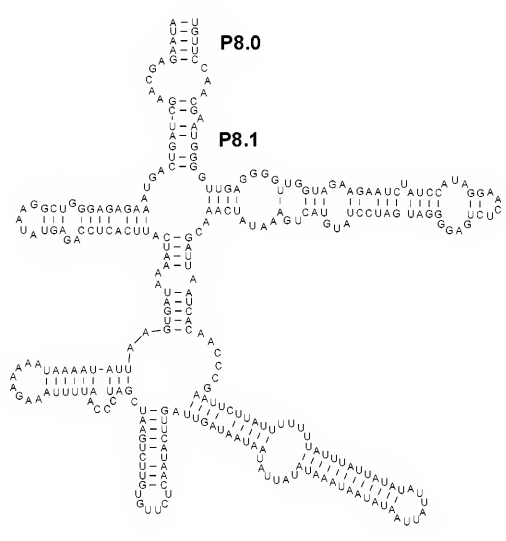
Lycopodium alpinum



Osmunda regalis



Gnetum gnemon



Nymphaea odorata

Figure 7. Variation of length and secondary structure of the stem-loop region P8 of the *trn*L_{UAA} intron.

stem-loop regions showed a considerable degree of compensating base pair changes among the sampled lineages (see Fig. 2). In addition, two different types of terminal loops in the stem-loop region P5 were observed, the first being A-rich and the second T-rich. Whereas the A-rich type was the more common, the T-rich type, which is probably the result of an inversion of the P5 loop, was found in a clade of eu- and leptosporangiate ferns, whisk ferns, and horsetails (Moniliformopses), as well as

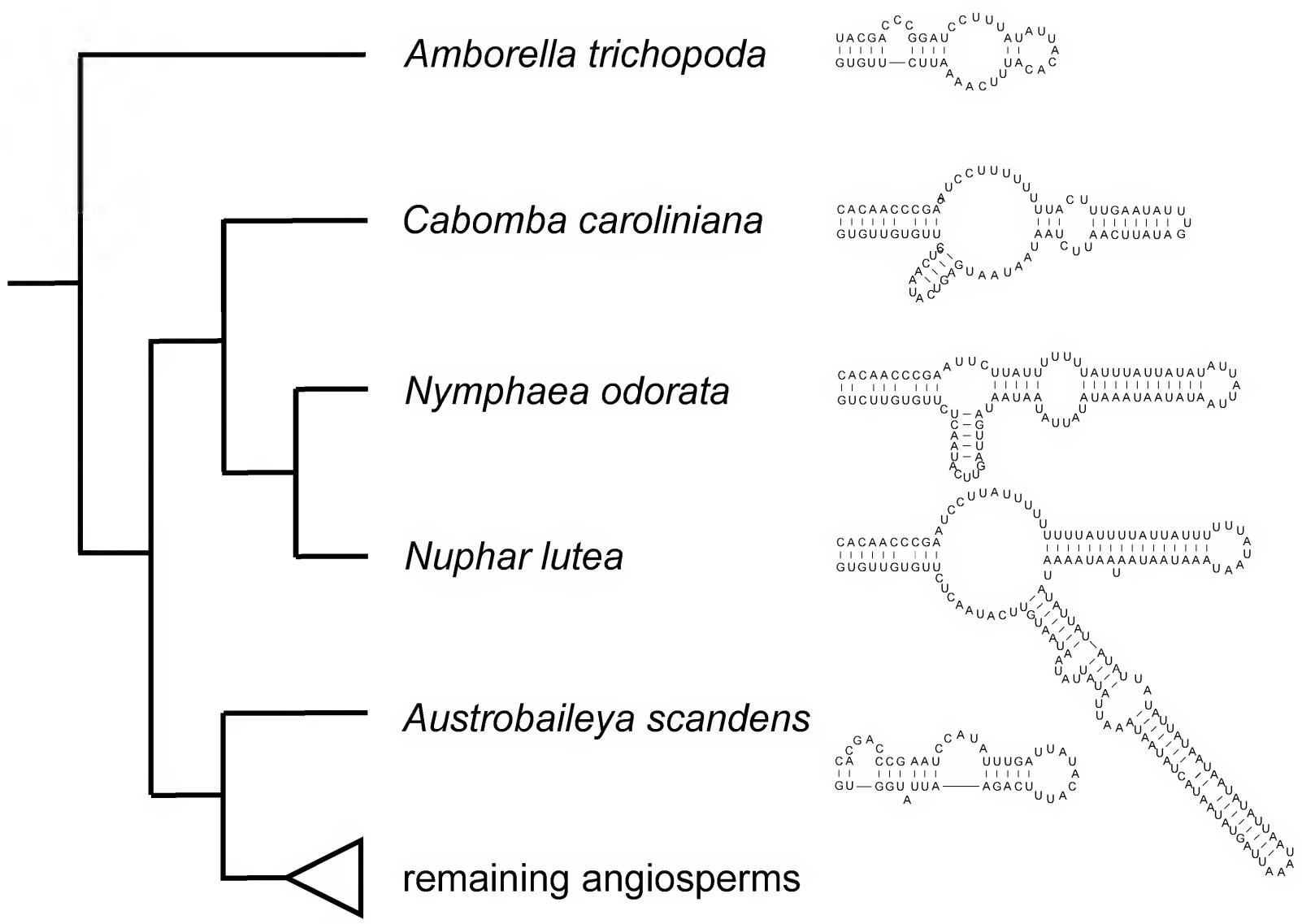


Figure 8. Summary of the current knowledge on the relationships among basal angiosperms extracted from Borsch et al. (2003), with the different P8 stem-loop regions being plotted on the tree.

in gymnosperms. Sequence and structural analyses also revealed two different types of P5 stems called A and B. Whereas type B is synapomorphic for seed plants apart from *Gnetum*, the remaining land plant lineages, including *Gnetum* as well as the green alga *Chlorella* and cyanobacteria, such as *Nostoc*, *Anacystis*, or *Phormidium* (Fig. 9; compare Kuhsel et al., 1990; Costa et al., 2002), are characterized by the type A. A notable exception is the stem-loop region P5 of the charophyte *Chaetosphaeridium* that differs from all other taxa by a 2 bp deletion of 5′ stem bases in P5 and thus shifting its helical structure (Figs. 2, 9). However, both predominant stem types (A and B) differed in a one-nucleotide shift of the corresponding nucleotides in the P5 helical element, due to two mutations (A to C and A to G), which more effectively stabilized the stem in type B (Fig. 9). In gymnosperms this shift reduced the 3′ internal loop to two nucleotides. Similarly, in angiosperms this 3′ internal loop also consists of two nucleotides, which, however, are not homologous to the ones in gymnosperms, as it seems that one of them has been inserted in angiosperms (compare Fig. 9). This insertion goes along with a mutation in P4. P4 in angiosperms differs considerably from that of other lineages, due to an A to G mutation (TCAGGG–TCAGAG) followed by a shift of the corresponding nucleotides in P4, further reducing the internal loop. The latter situation might have been compensated by an insertion of an extra nucleotide. This is supported by the fact that in some angiosperm species a back mutation to TCAGGG occurred (e.g., *Vitis riparia*) that had no effect on the inserted nucleotide or the P5 stem. As this insertion is located directly at the border of the conserved element P, and due to the fact that there are two alternatives of gap positioning, it has an impact on the phylogenetic signal as well as on the consensus of the conserved sequence element P (aligned positions, following Cech (1988) and Kuhsel et al. (1990), are shown in Table 3). The present sequence alignments combined with structural analyses, however, indicate that in angiosperms a single nucleotide was inserted in P, which at first glance during simple primary sequence comparisons remains obscured. This example also illustrates that the pattern of coevolving sites might be more complex, as corresponding nucleotides might differ over time due to helical shifts.

Similar to the core intron sites, the *trn*L_{UAA} gene was found to be rather conserved, with length mutations being very limited in number and confined

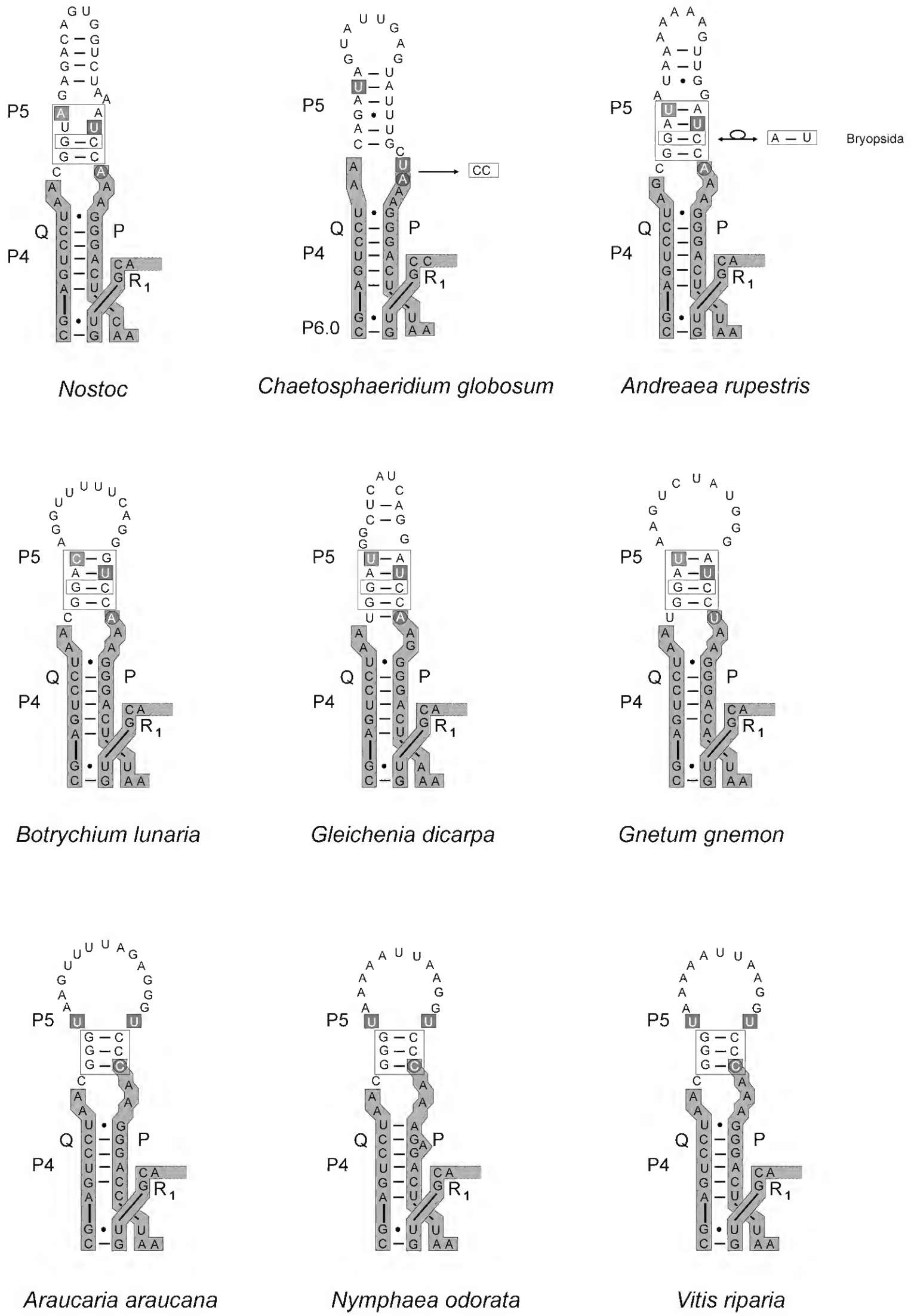


Figure 9. Secondary structure representations of the stem-loop region P4 and 5 of the *trn*L_{UAA} intron. See text for explanation.

Table 4. The impact of compensatory base pair changes on the phylogenetic structure R. d = difference between R when (i) all CBC regions are included (helical elements in P1–P9, excluding P8) and (ii) R when only one half out of two corresponding halves is included. s.e. = standard error. See text for further explanation.

	Mean d	s.e.	95% confidence interval
5′ helix strand	0.0398	0.0102	0.0197–0.0599
3′ helix strand	0.0231	0.0089	0.0057–0.0405

to the loop of the extra arm of the cloverleaf structure. The latter is completely built by the *trn*L_{UAA} 3′ exon (Fig. 2A). Compensating base pair changes were observed in both exons, and some examples are plotted on the cloverleaf structure shown in Figure 2A. In addition, a rather peculiar transition was observed in the anticodon site of the *trn*L_{UAA} gene in *Gingko* and the leptosporangiate ferns sampled (except for *Osmunda regalis*). This transition, changing the anticodon from UAA into CAA, had been previously observed in *Takakia* (Takakiopsida, Quandt & Stech, 2004) and *Adiantum capillus-veneris* (Wolf et al., 2003). The mutation is of special interest because it implies a further case of possible tRNA anticodon editing, which so far has been reported only once from the chloroplast genome (Kugita et al., 2003). A comparison with available sequence data in GenBank revealed that this transition also occurs in other leptosporangiate ferns such as in *Cibotium barometz* (AY142482), *Sphaeropteris brunoniana* (AJ505933), and *Alsophila spinulosa* (AJ505915). Hence, it seems that this transition unites the leptosporangiate ferns, except *Osmunda*.

THE *TRN*L-F SPACER

The *trn*T-F region consists of rather independent genes, which seem to be, at least in algae, monocistronically transcribed, but were placed next to each other after a series of reorganizations at the beginning of chloroplast genome evolution in land plants. Support for this scenario comes from the algal chloroplast genomes so far characterized, where the respective tRNA genes are remotely scattered. Chloroplast genomes of charophytes, a group of putative green algal ancestors of land plants, however, already seem to be quite similar to those of land plants, as shown by Turmel et al. (2002) by comparing *Chaetosphaeridium globosum* and *Marchantia polymorpha*. Both genomes share 12 blocks of collinear sequences, with up to 40 genes present in the same individual clusters. Differences in gene order are largely attributed to only 12 inversions with seven shared endpoints within or near tRNAs (Turmel et al., 2002). In the context of the *trn*T-L-F arrangement, it is noteworthy that one of the blocks that *Chaetosphaeridium* and *Marchantia* have in common is a cluster consisting of *rps*4, *trn*T, and *trn*L, whereas *trn*F is found in a different block approximately 20 kb downstream of *rps*4-*trn*T-*trn*L in *Chaetosphaeridium*, together with *ndh*J, *ndh*K, and *ndh*C. This leaves two possible explanations for the evolutionary history of the region. Either a common ancestor of charophytes and land plants already had the *trn*T-L-F arrangement and the region was divided again on the branch leading to the charophytes, or it is a true synapomorphy for land plants.

Although many tRNA genes contain sequences similar to the prokaryotic promoter in their upstream regions (see Wakasugi et al., 1986), it was rather surprising to observe a putative sigma^{70}-type bacterial promoter motif (σ-type; −35 TTGACA,—10 TAGGAT) in most of the sampled taxa approximately 45 bp upstream of the *trn*F_{GAA} gene, as *trn*F_{GAA} is supposed to be cotranscribed with *trn*-L_{UAA} (Kanno & Hirai, 1993). This motif was found almost consistently across land plants including angiosperms, although with various deviations, and had been previously noticed by van Ham et al. (1994). In particular, the motif is absent from lycophytes, leptosporangiate ferns, and Gnetales. The absence in the latter is discussed in detail by Won and Renner (submitted). As the promoter motif in front of *trn*F_{GAA} in land plants is very similar to the *trn*F_{GAA} promoter in *Chaetosphaeridium*, and considering the fact that the *trn*F-*ndh*J-*ndh*K-*ndh*C block was translocated next to or from *rps*4-*trn*T-*trn*L during the evolution of the chloroplast genome, we assume that the present promoter is the original *trn*F_{GAA} promoter that presumably is still active in green algae such as *Chlorella* or *Chaetosphaeridium*. Interestingly, the almost ubiquitous presence of this putative promoter implies that it might still serve some function in land plants. In most mosses, the *trn*L-F spacer almost exclusively consists of this putative promoter, which is able to form a stable hairpin with an occasionally inverted loop (Quandt & Stech, 2004). In addition, the enormous length variation across land plants is almost exclusively confined to the region between the putative promoter and the *trn*L_{UAA} 3′ exon. Similar to the P8 stem-loop region the *trn*L-F spacer displays a high sequence similarity within defined groups of taxa, where the region is alignable, such as in angiosperms or mosses, although it is impossible to align it across all land plants. Therefore, the as-

signment and definition of the mutational hotspots observed within the *trn*L-F region between different lineages varies considerably from each other (e.g., Borsch et al., 2003; Quandt & Stech, 2004; Won & Renner, submitted). However, in leptosporangiate ferns, lycophytes, and Gnetales (cf. Table 2; Won & Renner, submitted), the typical promoter motif could not be detected next to $trnF_{GAA}$, but was occasionally found elsewhere within the spacer, e.g., in *Huperzia squarrosa* or *Gleichenia dicarpa*.

As Wolf et al. (2003) pointed out, the chloroplast genome of leptosporangiate ferns such as *Adiantum capillus-veneris* differs from other land plant lineages considerably due to various reorganizations during its evolutionary history. Hence, it seems likely that the organization and composition of the *trn*L-F spacer has, apart from its sequence divergence over time, a different evolutionary history in each lineage. This is underscored by the high sequence divergence and length variation, attributed to frequent insertions and various repeats possibly due to slipped strand mispairing. In addition, breakpoints of chloroplast rearrangements seem to be associated with short indirect repeats found within or near tRNA genes (Turmel et al., 2002). Therefore, spacer regions between tRNAs might not be homologous across all land plant lineages due to reorganization. In fact, for leptosprangiate ferns the following scenario would also be possible: if the *trn*L-F region is a synapomorphy for land plants, and if the *trn*L promoter is able to control the expression of both *trn*L and *trn*F, the *trn*F gene could have lost its promoter motif via a double translocation. In this case, the promoter would have been rearranged together with the *trn*F gene. A second rearrangement would then be required to relocate only the *trn*F gene next to *trn*L. The loss of the promoter would not have been as problematic as the promoter in front of *trn*L would control *trn*F expression. Assuming such a scenario, the history of the spacer region would be very different from all other land plants, which might explain the extant high sequence divergence between leptosporangiate ferns and other land plant lineages. Based on the accumulated data (cf. Table 1), it is uncertain how the spacer was composed in first place, i.e., which 5′ and 3′ spacer sequences were contributed from their former locations. The distribution of different lengths throughout the phylogeny indicates that the spacer is rather short in more basal land plant lineages, such as liverworts and mosses (Figs. 3, 5). Therefore, it seems reasonable that the translocation placed the $trnF_{GAA}$ gene in close proximity to the $trnL_{UAA}$ 3′ exon, together with its promoter. Thereupon, elongations occurred independently, leading to different spacer sequences in the different lineages. Whereas in most land plants the elongation occurred between the $trnL_{UAA}$ 3′ exon and the putative promoter, it is not as clearly confined to a certain area in leptosporangiate ferns and lycophytes, which in addition might have lost the putative promoter.

Other causes of length variability in the *trn*L-*trn*F IGS are the insertion of partial or complete pseudogenes of *trn*F as has been observed in *Microseris* (Asteraceae, Vijverberg & Bachmann, 1999) or certain lineages of Brassicaceae (Dobes et al., 2004). These insertions seem to occur and to be homoplastic within defined lineages of angiosperms and are most likely to be explained by interchromosomal recombination with unequal crossing over (Vijverberg & Bachmann, 1999).

THE *TRN*L INTRON TREE OF LAND PLANTS

The inferred tree generally depicts a similar topology to the multi-gene analysis by Pryer et al. (2001), although a direct comparison is hampered due to missing algal outgroups in Pryer et al. (2001) and differences in taxon sampling, especially within angiosperms. However, although only a small fraction of the *trn*L intron sequences could be used for the phylogenetic inference the main land plant lineages—such as the bryophytes (in this case including liverworts, hornworts, and mosses), lycophytes, Moniliformopses, gymnosperms (excluding *Gnetum*), and angiosperms—are resolved monophyletic (Fig. 3). Interestingly, bryophytes including hornworts form a monophyletic group with hornworts being sister to liverworts, which is in contrast to various published trees (e.g., Mishler et al., 1994; Hedderson et al., 1996; Lewis et al., 1997; Duff & Nickrent, 1999). However, for a detailed discussion on the position of hornworts we refer to Stech et al. (2003a). Resolution within the Moniliformopses in our analysis is largely congruent with Pryer et al. (2001), although minor differences exist such as the position of the horsetails. The main difference to Pryer et al. (2001) is reflected in the position of *Gnetum* that is resolved as the basalmost seed plant in our analysis. Branch lengths generally tend to be very short, especially within angiosperms. Nevertheless, within basal angiosperms *Amborella* is resolved basalmost followed by the Nympheales, a placement that is congruent with Borsch et al. (2003) or Qiu et al. (1999, 2000).

IMPACT OF COMPENSATORY BASE PAIR CHANGES ON THE PHYLOGENETIC SIGNAL

Recently, Borsch et al. (2003) provided a well-resolved and highly supported tree for relationships

among basal angiosperms. However, especially on much deeper phylogenetic levels, such as among land plants, part of the phylogenetic signal may be contributed by non-independently but coevolving sites. This also relates to other regions being used especially at deeper phylogenetic levels that exhibit functional secondary structures on RNA level (cf. Soltis et al., 2000). Therefore, we performed tests to assess the impact of compensatory base pair changes on the phylogenetic structure present in the data set via the exclusion of information gained from one half of the putatively coevolving helices. Our test clearly showed that removing information gained from coevolving helices significantly decreases the phylogenetic structure, independent of which side of the helical element is being removed (Table 4). However, the signal provided by each "half" is not perfectly symmetrical, i.e., 5′ parts do not exactly duplicate information from 3′ parts (Table 4). This is probably due to imperfect matches, i.e., due to reversals or ambiguities. Moreover, shifts of corresponding partners such as documented for P5 might result in a non-symmetric signal of both halves. Violation of the required independence of characters may be a common practice in most phylogenetic studies, since the presence of non-independence is not usually obvious or assignable to particular sites. Moreover, the relative effect of such sites on the overall phylogenetic inference, particularly in data sets with relatively large number of parsimony informative characters, may commonly be irrelevant or at least assumed to be minor, such as in the case of Borsch et al. (2003). In contrast to the angiosperm dataset of Borsch et al. (2003), the conserved intron sites constitute large parts of the land plant data set analyzed here. Therefore, the presence and location of non-independent sites is rather evident, and a significant portion of the phylogenetic structure obtained from the data is likely to be gained from coevolving sites (CBCs). It has been observed that an inferred tree receives significantly less support or even collapses when only one out of two substitutional events of a CBC is used. However, if we look at their distribution, CBCs seem to be correlated with phylogenetic level. At the generic level almost no CBCs occur, whereas they become more frequent at deeper phylogenetic levels and are more likely to support deeper nodes (Quandt, 2002). This is underscored by the low sequence variability of the conserved core, which is partly illustrated by the highly conserved P, Q, R, and S consensus sequences. It should be pointed out that the inclusion of CBC characters can be regarded as a way of double-weighting characters. The question of whether an exclusion of at least one character out of each CBC-character pair should be favored is, therefore, arising. However, variability of the conserved helical elements will only matter at greater distances and thus be irrelevant for within clade analyses, such as within angiosperms. In deeper phylogenetics, such as among land plant lineages, down-weighting positions that correspond to coevolving helices in a phylogenetic analysis would be one way to deal with this complex topic. On the other hand, the affected characters might be phylogenetically very useful, as they have been fixed during evolution and could be generally less homoplasious.

Conclusion

Although the *trn*L_{UAA}–*trn*F_{GAA} region is thought to be a synapomorphy of land plants, it seems unlikely that all parts of the region share the same evolutionary history. Sequence and structural analyses point towards independent evolution of certain elements in stem-loop regions P6 and P8 as well as the *trn*L_{UAA}–*trn*F_{GAA} intergenic spacer in the different lineages. If cyanobacterial or green algal *trn*L_{UAA} introns are considered to be potential ancestors of the *trn*L_{UAA} intron in land plants, elongation and secondary losses of large parts of stem-loop regions P6 and P8 must have occurred several times independently. Inserted sequence parts can, therefore, be regarded as "internal paralogues" that arose and further differentiated in certain lineages. Structural analyses did not reveal a common secondary structure for the stem-loop region P8 across land plants. As a consequence for phylogenetic analysis, the *trn*L_{UAA} intron cannot be used in its full length for phylogenetic analyses at such deep levels. Sequence blocks of probably independently evolving elements need to be identified for the different land plant lineages and to be positioned next to each other in an alignment following their SSM-mediated origin. Afterwards, these blocks can be included in phylogenetic analysis to utilize substitutions within them. For seed plants this block comprises most of the length of P6 and P8. In contrast, the more conserved regions [PQRS] of the *trn*L intron are very similar, but prone to compensatory base pair changes. Compensatory base pair changes frequently observed in the more conserved part of the intron largely contribute to the phylogenetic signal. We showed that CBCs have a significant impact on the phylogenetic structure obtained from the *trn*L intron in the land plant data set, especially at deep phylogenetic levels. Such

effects might be true for any DNA region that exhibits a secondary structure on the RNA level.

Considering that the three tRNA genes contributing to the *trn*T-F region are more or less scattered in algal chloroplast genomes, it is reasonable to assume that each of the tRNAs is controlled by its own promoter in algae. Therefore, it seems likely that the putative σ-like promoter preceding *trn*F_{GAA} was translocated together with its gene. This is emphasized by the fact that in most bryophytes almost the complete *trn*L_{UAA}–*trn*F_{GAA} intergenic spacer except the putative σ-type promoter is missing. Length variation mainly occurs between the promoter and the *trn*L_{UAA} 3′ exon, with the exception of the leptosporangiate ferns and lycophytes, where the typical promoter motif could not be detected.

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PHYLOGENETIC RELATIONSHIPS OF THE HORNWORTS BASED ON *RBC*L SEQUENCE DATA: NOVEL RELATIONSHIPS AND NEW INSIGHTS[1]

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Abstract

A molecular phylogenetic analysis based on nucleotide sequence data from the plastid *rbc*L gene is presented for 20 species representing seven of eleven genera of hornworts (Anthocerotophyta). This analysis suggests hierarchical relationships that are not reflected in any current classification. Hornworts are divided into several genetically divergent groups. The first consists of a smooth-spored generic segregate *Leiosporoceros.* This sample forms the basal member of the hornwort clade and prior to this analysis was considered to be sister to, or a subgenus of, *Phaeoceros.* Further examination of the morphology of this genus supports the observed genetic isolation. The remaining hornworts are divided into two clades, one including *Anthoceros* plus *Folioceros* and the other containing the remainder of the genera. *Megaceros* plus *Dendroceros* form a monophyletic assemblage, a result that agrees with previous phylogenetic hypotheses. Additional departures from current classifications include the divergence of *Anthoceros* and *Phaeoceros,* a sister relationship between *Notothylas* and *Phaeoceros,* and polyphyly of *Phaeoceros* and *Dendroceros* and *Megaceros.* These results suggest novel yet intuitive relationships that in turn clarify the directionality of morphological change. For example, we can now postulate that morphology and habitat of the ancestral hornwort was like that of *Leiosporoceros, Anthoceros,* and *Phaeoceros.* Likewise, features found in *Megaceros, Dendroceros,* and *Notothylas* represent adaptations that result from radiations into more specialized habitats.

Key words: Anthocerotophyta, early land plant phylogeny, hornworts, *Leiosporoceros,* pyrenoid, *rbc*L, stomata.

In spite of a recent resurgence in interest in early plant phylogeny, the hornworts (Anthocerotophyta) have remained relatively unexplored, both at the molecular and morphological levels. Fundamental gaps still exist in our knowledge of hornwort biology. Morphological diversity has not been sufficiently characterized and taxonomic boundaries are highly controversial. Phylogenetic analyses, which are almost exclusively based on morphological data, have resulted in highly incongruent results (Renzaglia & Vaughn, 2000; Stech et al., 2003). Because of the pivotal role hornworts play in early embryophyte evolution, clarification of the primary branching patterns of land plants is untenable without a clear understanding of the genetic and morphological biodiversity within the group.

Many structural features of hornworts represent adaptations to life on land that are the products of deep divergences and thus are unparalleled in other groups of land plants (Renzaglia et al., 2000). Most notable are the internal sequestration of vulnerable reproductive structures, the copious production of mucilage, and the peculiar structure of an elaborate sporangium/sporophyte that elongates and continuously produces spores throughout the growing season (Renzaglia & Vaughn, 2000). Combined with plesiomorphic traits such as the presence of a large solitary chloroplast with a central pyrenoid (Vaughn et al., 1992) and presumably derived features such as stomata, the structural complexity of hornworts provides evidence for opposing views on the position of this ancient lineage among modern-day plants. Consequently, the hornworts have played a central role in recent debates over

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early land plant evolution. Specifically, the controversy focuses on whether the hornworts or liverworts were the first extant embryophyte lineage to diversify. Even with careful analysis of molecular and morphological markers, ambiguity still exists in regards to the basalmost land plant group (Garbary et al., 1993; Mishler et al., 1994; Kenrick & Crane, 1997; Qiu et al., 1998). The reasons for this dilemma are numerous and include an alleged rapid radiation of early plants on land, poor fossilization of fragile bryophyte tissues, inadequate taxon sampling, insufficient knowledge of morphological variability, and a lack of understanding of molecular diversity and evolution within bryophytes.

While hornworts clearly represent a monophyletic group, attempts at clarifying hornwort taxonomy and classification have yielded highly variable results. Of the 11 named hornwort genera, 6 have garnered wide support: *Phaeoceros*, *Megaceros*, *Dendroceros*, *Anthoceros*, *Notothylas*, and *Foliocer-os*. Table 1 shows four classification schemes proposed over the past 15 years. These classifications show no consensus in ordinal or familial ranking of taxa, and even at the generic level little agreement exists. In general, *Phaeoceros* and *Anthoceros* are viewed as sister taxa due to similarities in habit and sporophyte anatomy. Similarly, a sister relationship between *Megaceros* and *Dendroceros* is suggested by shared diagnostic features including spiraled elaters and the lack of stomata. Beyond these relationships, no common taxonomic interpretations are evident in these schemes.

Until recently the hornworts were the only major group of land plants for which a molecular study had yet to been undertaken at any taxonomic level. Stech et al. (2003) reported a molecular phylogeny that included nine hornwort sequences of the *trn*L-*trn*F chloroplast spacer. Their study focused on the position of the group among basal plants. Here we present a broader molecular phylogenetic analysis of hornworts based on nucleotide sequence data from the plastid *rbc*L gene. With more extensive taxon sampling, including additional species of the five genera included in the *trn*L study and representatives of three other genera (*Leiosporoceros*, *Folioceros*, and *Nothoceros*), the *rbc*L analysis presented here represents the first comprehensive molecular analysis of hornwort phylogeny. This phylogenetic hypothesis reveals intriguing, new interrelationships among genera and challenges traditional views of hornwort affinities. When morphology and ultrastructural characters such as chloroplast and sporophyte anatomy are examined, these novel relationships are highly intuitive and new insights on character evolution are revealed.

Materials and Methods

Nomenclature and sampling methods

No single taxonomic study has described species across the geographical range of our sampling; hence, we have followed several taxonomic treatments in assigning generic and species names, most notably that of Hasegawa (1994) and Hässel de Menéndez (1988). For most samples we have gathered live fruiting plants for identification and DNA isolation. Herbarium vouchers have been made of all live material and are deposited in CANB and PMA, with duplicates lodged at MO or ABSH. A list of hornwort samples, voucher information, and GenBank accession numbers is provided in Table 2. Because of the debate over potential outgroup taxa (Hedderson et al., 1996; Kenrick & Crane 1997; Lewis et al., 1997; Qiu et al., 1998; Parkinson et al., 1999; Nickrent et al., 2000), we have chosen to root the hornwort phylogeny with representative sequences of mosses, liverworts, green algae, and lycophytes.

DNA extraction and DNA sequencing

Total DNA was extracted from either living or dried material using the DNeasy Plant Kit (QIAGEN). Amplification of the *rbc*L gene was accomplished using primers designed to take advantage of hornwort specific mutations in the 5′ and 3′ end of the *rbc*L gene. These primers (rbcLHF 5′-GTCACCACAAACGGARACTAAAGC-3′ and rbcLHR 5′-CTTTCCATACTTCRCAAGCAGC-3′) anneal to base pairs 17–40 and base pairs 1393–1404 of *rbc*L from *Marchantia polymorpha* (Ohyama et al., 1986). Typically, PCR products were cloned into the TOPO TA sequencing vector (Invitrogen) prior to sequencing. Plasmid DNAs were sequenced from both directions in 10 μl reactions using both universal plasmid primers and a combination of internal sequence primers (rbcL471F 5′-CAAGGTCCACCTCATGGTA-3′, rbcL946F 5′-ACACGAAAGTGAATACCATG-3′, rbcL660R 5′-AACGATCTCTCCAACGCA-3′, and rbcL1031R 5′-ACTTCACRTTCTCCCTGT-3′). Sequencing was accomplished using the BigDye version 2 sequence mixture and run on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems) at the University of Akron.

Sequence data were initially edited and aligned using the alignment program SeqApp (Gilbert, 1993). Sequences for 24 outgroup taxa were obtained from GenBank and aligned with newly generated hornwort sequences (Table 2). All sequences were unambiguously aligned with only a single

Table 1. Recent taxonomic history of Anthocerotophyta. Ordinal names appear in boldface. Authorities as follows: [a] Limpricht in Cohn; [b] Dumort.; [c] (Mich.) L., emend. Prosk. 1951; [d] (Milde) Müll. Frib. ex Prosk. emend. Hässel; [e] Sull.; [f] Prosk.; [g] D. C. Bharadwaj; [h] Hässel; [i] (Milde) corr. and emend. Hässel; [j] Nes.; [k] Campbell; [l] Steph. emend. Rink; [m] R. M. Schust.; [n] Grolle; [o] Piippo; [p] (J. Haseg.) J. Haseg.; [q] (R. M. Schust.) J. Haseg.

Hässel de Menéndez (1988)	Schuster (1992)	Hyvönen and Piippo (1993)	Hasegawa (1994)
Anthocerotales[a]	**Anthocerotales**	**Anthocerotales**	**Anthocerotales**
Anthocerotaceae[b]	Anthocerotaceae	Anthocerotaceae	Notothyladaceae
Anthoceros[c]	subfam. Anthocerotoideae	*Anthoceros*	*Notothylas*
Sphaerosporoceros	**Aspiromitus*[l]	*Folioceros*	Anthocerotaceae
Notothyladaceae[d]	subgen. *Aspiromitus*	*Leiosporoceros*	subfam. Anthocerotoideae
subfam. Notothyladoideae	subgen. *Folioceros*	*Mesoceros*[o]	*Anthoceros*
Notothylas[e]	*Anthoceros	*Phaeoceros*	subgen. *Anthoceros*
subfam. Phaeocerotoideae	subgen. *Anthoceros*	*Sphaerosporoceros*[h]	subgen. *Folioceros*
Phaeoceros[f]	subgen. *Leiosporoceros*	Dendrocerotaceae	*Leiosporoceros*
Foliocerotales	subfam. Dendrocerotoideae	*Dendroceros*	*Hattorioceros*[p]
Foliocerotaceae	*Megaceros*	*Megaceros*	*Phaeroceros*
Folioceros[g]	subgen. *Megaceros*	**Nothothylales**	subfam. Dendrocerotoideae
Leiosporocerotales	subgen. *Nothoceros*	Notothyladaceae	*Dendroceros*
Leiosporocerotaceae[h]	*Dendroceros*	*Notothylas*	subgen. *Dendroceros*
Leiosporoceros[h]	subgen. *Dendroceros*		subgen. *Apoceros*
Dendrocerotales	subgen. *Apoceros*[m]		*Megaceros*
Dendrocerotaceae[i]	subfam. Notothyladoideae[n]		*Nothoceros*[q] [*Notoceros* in error]
Dendroceros[j]	*Notothylas*		
Megaceros[k]			

* This author recognizes *Aspiromitus* in place of *Anthoceros* and *Anthoceros* in place of *Phaeoceros* (Stotler & Crandall-Stotler, 2003).

Table 2. Collection localities of hornwort species and GenBank accession numbers of their *rbc*L gene sequences. Vouchers of specimens have been placed in CANB. Asterisks denote outgroup samples used in the analysis presented in Figure 1. Numbers in parentheses distinguish multiple samples of the same species.

Taxon	Voucher	GenBank accession no.
INGROUP		
Megaceros		
M. sp. indet. 1	Australia, Queensland, *D. C. Cargill 14*	AY463035
M. flagellaris (Mitt.) Steph.	Australia, Queeensland, *D. C. Cargill 103*	AY463036
M. gracilis (Reichardt) Steph. (1)	Australia, New South Wales, *S. Donaldson 2552*	AY463037
M. denticulatus (Lehm.) Steph.	New Zealand, *Duckett s.n.*	AY463038
M. gracilis (2)	Australia, Victoria, *B. A. Fuhrer s.n.*	AY463039
M. flagellaris	New Zealand, *Duckett s.n.*	AY463040
M. pellucidus (Colenso) E. A. Hodgs.	New Zealand, *Duckett s.n.*	AY463041
M. sp. indet. 2	Costa Rica, Tapantí, *Salazar Allen et al. 20641*	AY463044
M. aff. *vincentianus* (Lehm. & Lindenb.) Campb.	Panamá, El Copé, *J. C. Villarreal 641*	AY463045
M. aenigmaticus R. M. Schust.	U.S.A., Tennessee, Manhart (1994)	L13481
Nothoceros		
N. giganteus (Lehm. & Lindenb.) J. Haseg.	New Zealand, *Duckett s.n.*	AY463046
Phaeoceros		
P. coriaceus (Steph.) E. O. Campb.	New Zealand, *Duckett s.n.*	AY463042
P. hirticalyx (Steph.) J. Haseg.	New Zealand, *Duckett s.n.*	AY463043
P. carolinianus (Michx.) Prosk. (1)	*in* Parkinson et al. (1999)	AF244562
P. carolinianus (2)	U.S.A., Illinois, Murphysboro, *K. S. Renzaglia 3001*	AY463057
P. carolinianus (3)	Panamá, El Valle Antón, *Villarreal & Varela 520*	AY463058
P. sp. indet.	Australia, Victoria, *D. C. Cargil 363*	AY463056
Dendroceros		
D. aff. *canaliculatus* Pagán	Panamá, Cerro Pelado, *Villarreal & Varela 584*	AY463047
D. crispatus (Sw.) Nees	Australia, *D. C. Cargil 28*	AY463048
D. granulatus (Mitt.) Steph.	New Zealand, *Duckett s.n.*	AY463049
D. sp. indet.	*in* Newton et al. (2000)	AF231888
Notothylas		
N. breutelii (Gottsche) Gottsche	U.S.A., Louisiana, *D. & S. Krayeski,* cultured	AY463054
N. orbicularis (Schwein.) Sull.	U.S.A., Illinois, Murphysboro, *K. S. Renzaglia s.n.*	AY463055
Folioceros		
F. fuciformis (Mont.) D. C. Bhardwaj (1)	Singapore, *B. Tan s.n.*	AY463050
F. fuciformis (2)	Australia, Queensland, *A. Cairns s.n.*	AY463051
Leiosporoceros		
L. dussii (Steph.) Hässel	Panamá, El Valle de Antón, *Villarreal & Araúz 651*	AY463052
Anthoceros		
A. laminiferus Steph.	New Zealand, *Duckett s.n.*	AY463053
A. punctatus L. (1)	U.S.A., North Carolina, Lewis et al. (1997)	U87063
A. punctatus (2)	*in* Nishiyama & Kato (1998)	AB013669
A. formosae[1] Steph.	*in* Kugita et al. (2003)	NC_004543
OUTGROUP		
*Isoetes melanopoda** Gay & Durieu	Manhart (1994)	L11054
Selaginella deflexa Brack.	Korall & Kenrick (2002)	AF093253
Huperzia verticillata (L. f.) Trevis.	Wikström & Kenrick (2000)	AJ133897
*Lycopodiella caroliniana** (L.) Pic. Serm.	Wikström & Kenrick (2000)	AJ133261
*Marchantia polymorpha** L.	Shimoda & Sugiura (1991)	NC_001319
Jubula pennsylvanica (Steph.) A. Evans	Lewis et al. (1997)	U87074
Fossombronia foveolata Lindb.	Lewis et al. (1997)	U87069

Table 2. Continued.

Taxon	Voucher	GenBank accession no.
Metzgeria furcata (L.) Corda	Lewis et al. (1997)	U87081
Monoclea gottschei Lindb.	Lewis et al. (1997)	U87083
*Ricciocarpos natans** (L.) Corda	Lewis et al. (1997)	U87089
*Sphaerocarpos texanus** Austin	Lewis et al. (1997)	U87090
Takakia lepidozioide *S. Hatt. & Inoue	Parkinson et al. (1999)	AF24565
*Sphagnum palustre** L.	Newton et al. (2000)	AF231887
Andreaea rupestris Hedw.	Magombo (2003)	AF478199
Polytrichum commune Hedw.	Lewis et al. (1997)	U87087
Atrichum altecristatum (Renauld & Cardot) Ireland	Magombo (2003)	AF478209
Funaria hygrometrica Hedw.	Magombo (2003)	AF226818
Tetraphis pellucida Hedw.	Magombo (2003)	AF478203
Mnium cuspidatum Hedw.	Lewis et al. (1997)	U87082
Andreaeobryum macrosporum Steere & B. M. Murray	Mishler et al. (unpublished)	AF231059
Chara vulgaris L.	McCourt et al. (1999)	AF097167
*Chara polyacantha** A. Braun	Sanders et al. (2003)	AY170453
Coleochaete pulvinata Pringsh.	Delwiche et al. (2002)	AY051142
*Coleochaete orbicularis** Pringsh.	Manhart (1994)	L13477

[1] *A. angustus* Steph. (Ashtana & Srivastava, 1991).

three base pair gap in one taxon, *Takakia*. The final alignment including all outgroup taxa was imported into PAUP* 4.0b8 and is available in NEXUS file format (Maddison et al., 1997) from the authors upon request.

Maximum parsimony and maximum likelihood analyses were performed using PAUP* version 4.0b8 (Swofford, 2001) on a Macintosh G4 PowerBook. We first employed maximum parsimony analysis on the larger data set including all 54 samples. Subsequent analyses were performed on a smaller data set that retained all of the ingroup taxa but included a smaller number of outgroup representatives. In these preliminary analyses all characters were unordered, given equal weight, and the following specifications were set: heuristic search, starting tree by stepwise addition, random addition sequence with 1000 replicates, random starting seed number, TBR branch swapping, MulTrees, and steepest descent. Subsequent searches implemented a step matrix of user-defined character types that included using only first and second base positions and the elimination of T/C transitions as a simple method of reducing the possible phylogenetic effects of RNA editing (Bowe & dePamphilis, 1996; Szmidt et al., 2001). The maximum likelihood criterion was applied only to the reduced data set of 39 taxa. These analyses were based on the HKY85 model with empirical base frequencies from the ingroup only and ML trees were constructed with settings as listed above for the maximum parsimony analysis. The credibility of inferred clades on the parsimony tree was tested by a bootstrap analysis (Felsenstein, 1985) with 10,000 replications with parsimony settings as above. Decay indices were calculated using AutoDecay 4.0.2 (Eriksson, 1999).

MORPHOLOGICAL STUDIES

All specimens were examined with the light microscope, photographed, and identified to species where possible. Live samples were prepared for examination in the transmission electron and scanning electron microscopes following standard protocols (Renzaglia et al., 2002). Images presented here are incorporated into the discussion as a means of illustrating diversity and character evolution within the group. Detailed anatomical and ultrastructural studies are ongoing and will be presented elsewhere.

RESULTS

*RBC*L ANALYSIS

The full data set consisted of 1336 bp for 30 ingroup taxa and 24 outgroup taxa. No gaps in the alignment were necessary except for a three base deletion present in the moss *Takakia lepidozioides*. Maximum parsimony analysis yielded 20 equally most parsimonious trees of 2549 steps (consistency index (CI) = 0.431, retention index (RI) = 0.723, uninformative characters excluded). A strict con-

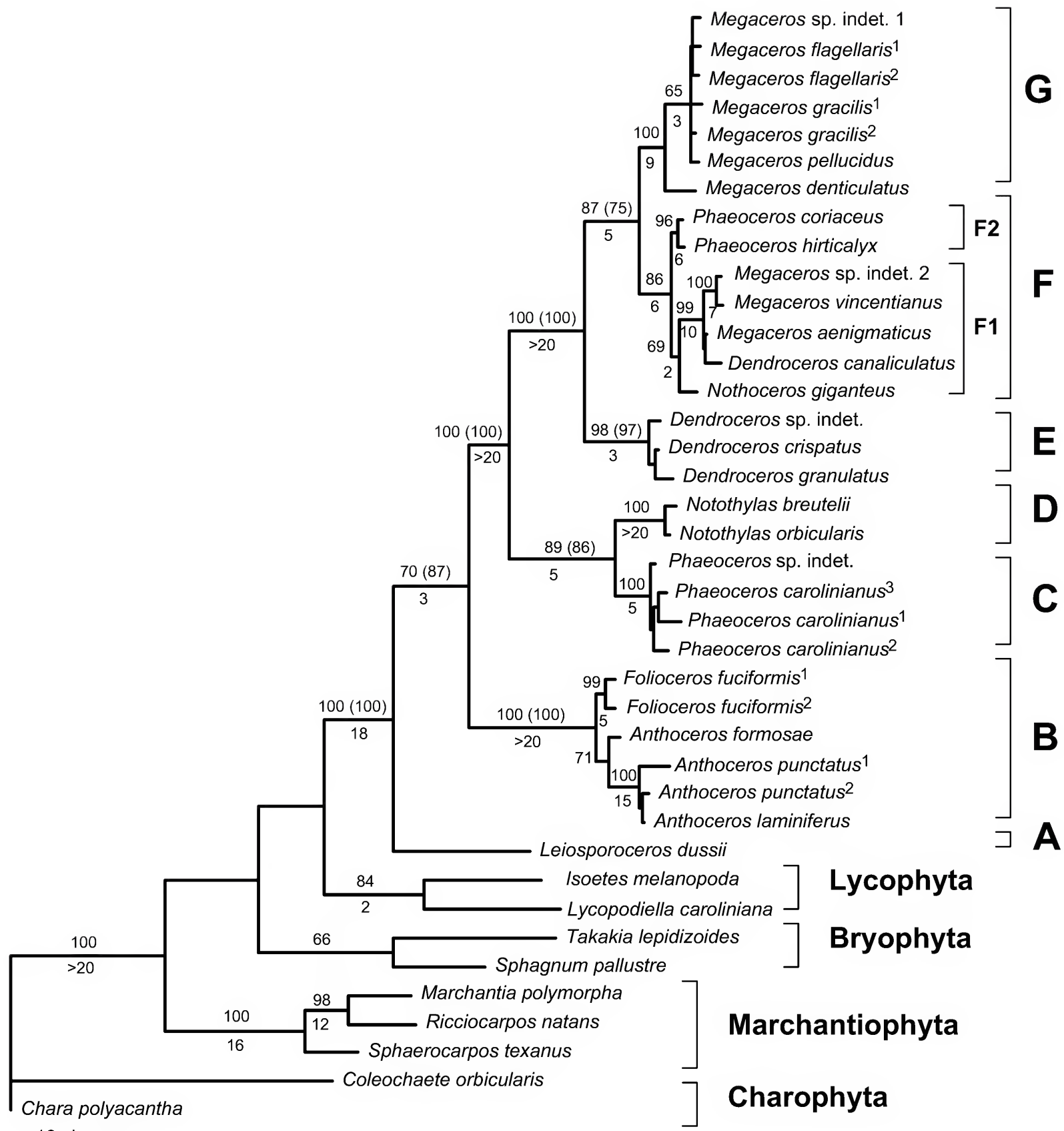

Figure 1. One of 10 most parsimonious trees. Values above branches denote bootstrap values (1000 replications) with 9 outgroups. Numbers in parentheses at selected nodes represent bootstrap values when all 24 outgroup taxa are used in the analysis. Numbers above branches are bootstrap values when the 9 outgroup taxa shown are used in the analysis. Numbers below branches denote decay indices. Branches with no values have decay indices of 2 or below and bootstrap values less than 50%. Superscript numbers indicate samples as identified in Table 2.

sensus tree of this analysis yields the topology in which liverworts are the basal land plant lineage with mosses paraphyletic to a hornwort-tracheophyte clade. Support for this topology is very weak with only the basal liverwort branch receiving greater than 50% bootstrap support. Although the relationship of hornworts to other land plants is ambiguous, the monophyly of hornworts is strongly supported (bootstrap 100%) and a number of clades within the hornworts are also well supported. As a result, we reduced the number of outgroup taxa (see Fig. 1) to 9 from 24, allowing more rigorous examination of the ingroup topology.

Maximum parsimony analysis of the reduced data set resulted in 10 most parsimonious trees with a shortest length of 1473, CI = 0.591 and RI = 0.780 excluding uninformative characters. One of these trees is shown as a phylogram with bootstrap and decay support indicated (Fig. 1). Maximum likelihood analyses resulted in a tree (not shown)

Table 3. Bootstrap support (1000 replications) for selected clades of hornworts as the result of parsimony analyses with various outgroup combinations (topologies not shown). All analyses were conducted with the full set of hornworts and no weighting of characters. The *Dendroceros/Megaceros* clade (E, F, and G from Fig. 1), clades B, C, and D had 100% bootstrap support for all analyses.

Outgroup	Hornworts	Hornworts–*Leiosporoceros*[1]	*Megaceros*[2]	*Dendroceros*[3]	*Notothylas* and *Phaeoceros*[4]
All outgroups	100	70	87	98	89
Liverworts only	100	80	79	84	78
Mosses only	100	97	80	86	90
Lycophytes only	100	52	83	91	78
Charophytes only	100	86	81	84	88
Liverworts and mosses	100	93	81	85	86
Mosses and lycophytes	100	80	78	80	82

[1] Clades B–F from Figure 1.
[2] Clades F and G from Figure 1.
[3] Clade E from Figure 1.
[4] Clades C and D from Figure 1.

with the identical topology to one of the 10 most parsimonious trees. Parsimony analyses of alternative data sets including the elimination of third base positions, weighting of transversions over transitions and using only transversion and G/A transition substitutions resulted in topologies (not shown) that were consistent among major clades though more poorly resolved at higher nodes.

IDENTIFICATION OF MAJOR CLADES OF HORNWORTS

Examination of the results from Figure 1 reveals hornworts as a strongly supported monophyletic group. The hornworts can be divided into seven clades. The first contains the taxon *Leiosporoceros*, which appears to be genetically isolated from, and sister to, all other hornworts (Fig. 1, Clade A). The second (Fig. 1, Clade B), consists of members of *Anthoceros* s.l. (*Anthoceros* + *Folioceros*) (Schuster, 1992). A large assemblage that contains all the other genera of hornworts includes the remaining five well-supported groups (Fig. 1, Clades C–G).

OUTGROUP COMPARISONS

To assess the influence of various outgroup taxa on the internal hornwort topology, we explored a number of potential outgroup combinations using the *rbc*L data set. These data (Table 3) show that higher clades among the hornworts are maintained regardless of which outgroup taxa were selected (topologies not shown). Outgroup selection, however, does appear to affect the support of *Leiosporoceros* as sister to all the hornworts.

MORPHOLOGY

An orbicular growth form with elongated sporophytes characterizes most hornwort genera (Fig. 2A). Gametophytes of *Dendroceros* are elongated with a distinct midrib and wings (Fig. 2B). *Notothylas* differs from other hornwort taxa in that sporophytes are determinate in growth, remaining surrounded by gametophytic tissue (Fig. 2C). Diversity is illustrated by comparisons of spores and pseudoelaters (Fig. 3), chloroplasts (Fig. 4), sporophyte anatomy (Fig. 5), and sporophyte epidermis (Fig. 6) among the taxa examined in this study.

Discussion

*RBC*L AND LAND PLANT PHYLOGENY

The *rbc*L analysis strongly supports the monophyly of hornworts but fails to resolve the affinities of hornworts with other plant lineages. The latter was not unexpected at this level of deep divergence as prior studies have shown that third-base pair positions are saturated, yet if removed leave too few characters to produce supportable resolution (Lewis et al., 1997; Kallersjo et al., 1998; Nickrent et al., 2000). Despite the limited use of *rbc*L for resolving deep branches in land plant evolution, the strong support of internal nodes in the *rbc*L tree (Fig. 1) demonstrates the potential of this gene for inferring phylogenetic relationships among hornworts. Indeed, regardless of weighting schemes or outgroup choice, this data set yields rather unambiguous relationships within hornworts (Table 3). Although additional sequence information from other sources will be required to confirm the *rbc*L topology for all the relationships, we believe that these results represent a significant step in inferring the phylogeny of hornworts and hence for assessing hornwort classification schemes and hypotheses of hornwort evolution.

Figure 2. Diversity in growth forms among hornworts. —A. *Anthoceros punctatus* L. Small orbicular gametophyte with both immature and almost ripe sporophytes, growing on soil. Involucres surround the base of sporophytes. Bar = 2 mm. D. C. Cargill, South East Gippsland, Victoria, Australia. —B. *Dendroceros crispatus* (Hook.) Nees. Sterile specimen growing as an epiphyte on bark; note the swollen midrib and monostromatic wings. Bar = 3 mm. Andi Cairns, Paluma, North Queensland, Australia. —C. *Notothylas orbicularis* (Schwein.) Sull. Small orbicular gametophytes growing on bare soil; note the numerous small, horizontally oriented sporophytes enclosed within involucres. Bar = 1 mm. A. J. Long, Cove Hollow, Illinois. Picture by S. Schuette.

*RBC*L PHYLOGENY YIELDS SURPRISING RESULTS

The *rbc*L phylogeny suggests hierarchical relationships that are not reflected in any current classification. Of the four classifications presented above (Table 1), all show significant departures from the overall topology, but each agrees in part with specific relationships supported by the *rbc*L phylogeny. *Leiosporoceros* (Fig. 1, Clade A) is highly divergent from other hornworts and appears to be the basalmost hornwort taxon. The basal position of *Leiosporoceros* in the *rbc*L phylogeny is retrieved in all analyses of the data; the degree of support depends on outgroup selection, with mosses providing the greatest support (bootstrap 97%, Table 3). One important caveat in regard to these results must be emphasized. The *Leiosporoceros rbc*L sequence is unique among hornworts in that it appears to lack RNA editing sites (Duff, unpublished). All other hornwort sequences in the data set are known or predicted to have 16 to 28 edited positions (Yoshinga et al., 1996; Duff, unpublished). The putative lack of editing of the *Leiosporoceros rbc*L transcript accounts for a large portion of the genetic isolation of this taxon, as these sites are almost all found in first- and second-base codon positions. Although the presence of RNA editing does not necessarily result in problems for phylogenetic analyses (Bowe & dePamphilis, 1996; Szmidt et al., 2001), the contrast of highly edited transcripts among all the samples except one serves to accentuate the genetic divergence of this single sample, thus increasing the potential for long branch attraction problems (Felsenstein, 1985). These effects may exaggerate support for the basal position of *Leiosporoceros.* In fact, preliminary analyses of mitochondrial *nad*2, *nad*5, and SSU sequences and nuclear 18S SSU sequences are equivocal in their placement of *Leiosporoceros* as sister to the hornworts, although

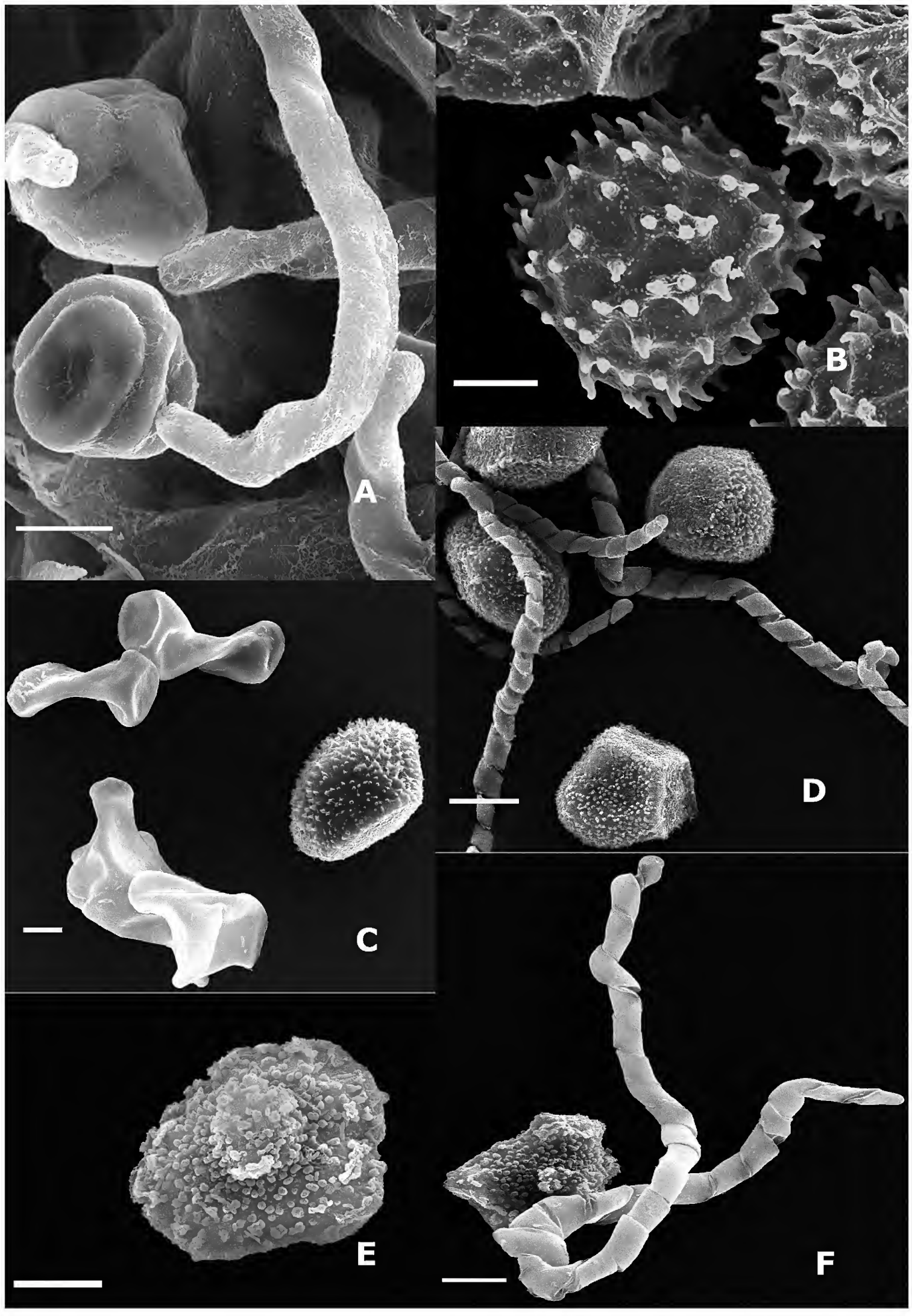

Figure 3. Scanning electron micrographs showing spore and pseudoelater diversity among hornworts. —A. *Leiosporoceros dussii* (Steph.) Hässel. Isobilateral tetrads containing small smooth spores among slightly spiraled thickened pseudoelaters. Bar = 10 μm. *Villarreal & Araúz 651* (CANB). —B. *Anthoceros punctatus* L. Spores showing both spiny distal face (middle and bottom right) and portions of the proximal face (upper left and right). Bar = 10 μm. —C. *Phaeoceros carolinianus* (Michx.) Prosk. Short pseudoelaters and a papillate spore in side view exposing mostly the distal face. Bar = 10 μm. —D. *Dendroceros crispatus* (Hook.) Nees. Multicellular spores and spiraled pseudoelaters. Bar = 20 μm. —E. *Megaceros gracilis* (Reich.) Steph. Distal face of spore showing central mammilla and small granules. Bar = 10 μm SD 2552 (CANB). —F. *Megaceros flagellaris* (Mitt.) Steph. Spiraled pseudoelater with spore in distal view. Bar = 10 μm.

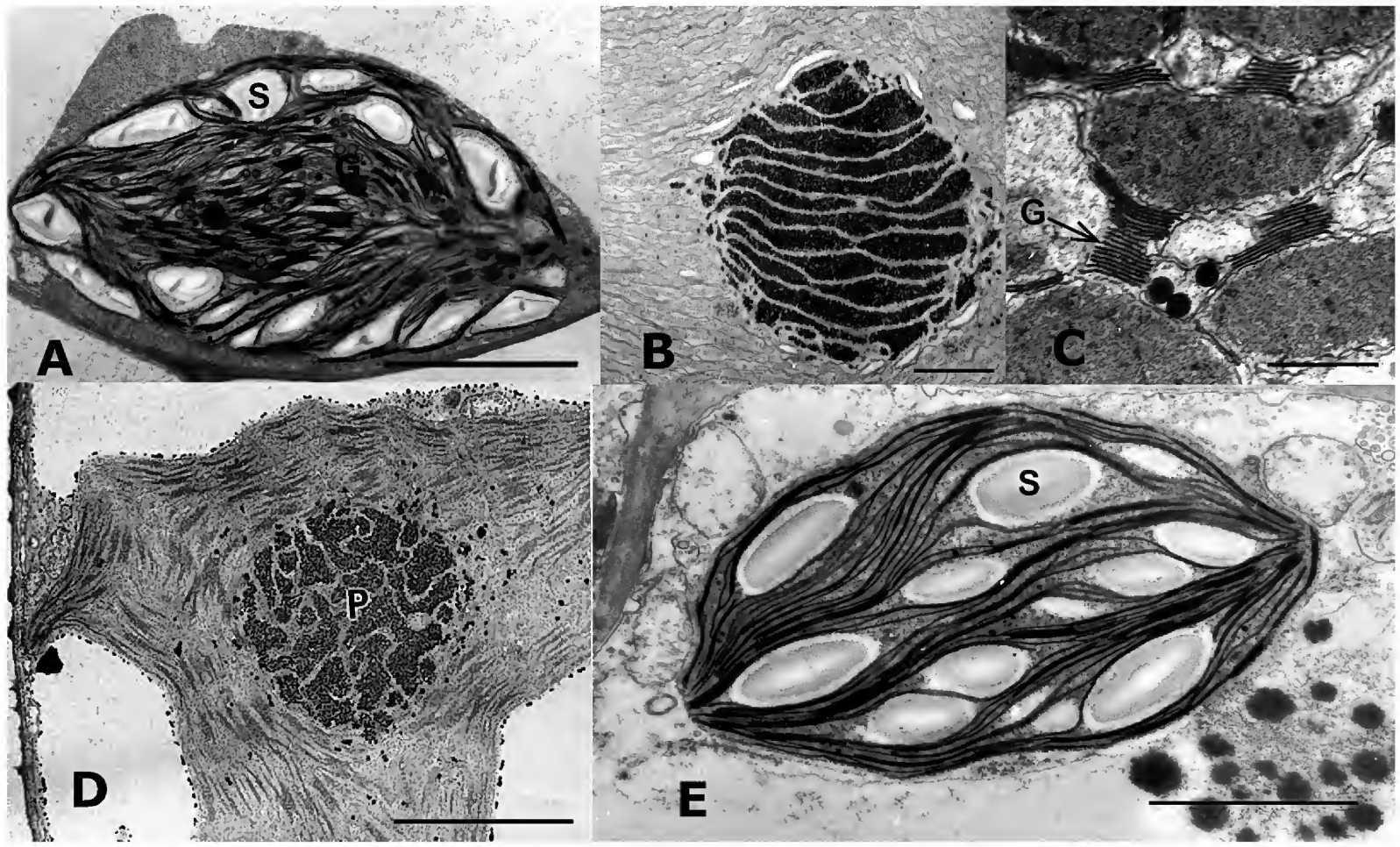

Figure 4. Transmission electron micrographs showing chloroplast diversity among hornworts. —A. *Leiosporoceros dussii* (Steph.) Hässel. Whole chloroplast showing peripheral starch (S) and centrally localized grana (G). Bar = 10 μm. *Villarreal & Araúz 651* (CANB). —B. *Notothylas breutelii* (Gottsche) Gottsche. Spherical pyrenoid with lens-shaped to elongated subunits containing uniform electron-dense inclusions and traversed by thylakoids. Small starch grains surround the pyrenoid. Bar = 2 μm. —C. *Anthoceros laminiferus* Steph. Grana (G) that lack end membranes separating lens-shaped pyrenoid subunits. Bar = 0.5 μm. *Duckett s.n.* (CANB). —D. *Dendroceros tubercularis* S. Hatt. Low magnification of a plastid revealing the irregular chloroplast outline and a central pyrenoid (P) that is highly dissected into irregular subunits. Bar = 5 μm. —E. *Megaceros* cf. *vincentianus* (Lehm. & Lindb.) Campb. Low magnification of a chloroplast with no pyrenoid and randomly distributed starch. Bar = 5 μm. *Villarreal 641* (CANB).

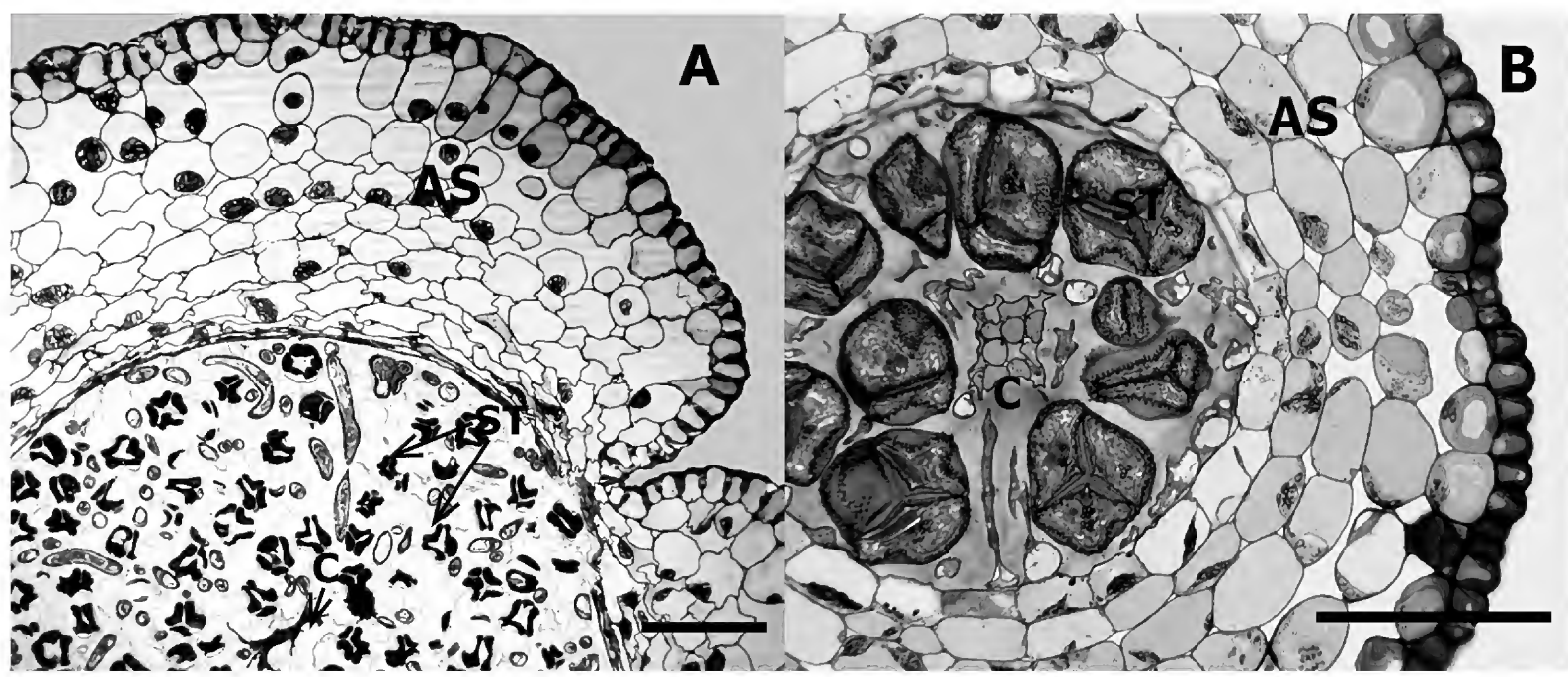

Figure 5. Light micrographs showing sporophyte anatomy in cross section. —A. *Leiosporoceros dussii* (Steph.) Hässel. Tissue is differentiated from outside to inside as follows: single layered epidermis, 9 to 10 layers of assimilative cells (AS), abundant sporogenous tissue with several layers of tetrads (ST) intermixed with pseudoelaters, and an indistinct columella (C). The suture is clearly defined as a longitudinal groove that extends nearly to the sporogenous tissue (lower right). Bar = 100 μm. *Villarreal & Araúz 651* (CANB). —B. *Anthoceros laminiferus* Steph. In contrast to Fig. 5A, this sporophyte contains an assimilative zone (AS) of five cell layers, sporogenous tissue with one or two layers of large tetrads (ST) intermixed with small pseudoelaters, and a columella (C) of 16 cells. Bar = 100 μm. *Duckett s.n.* (CANB).

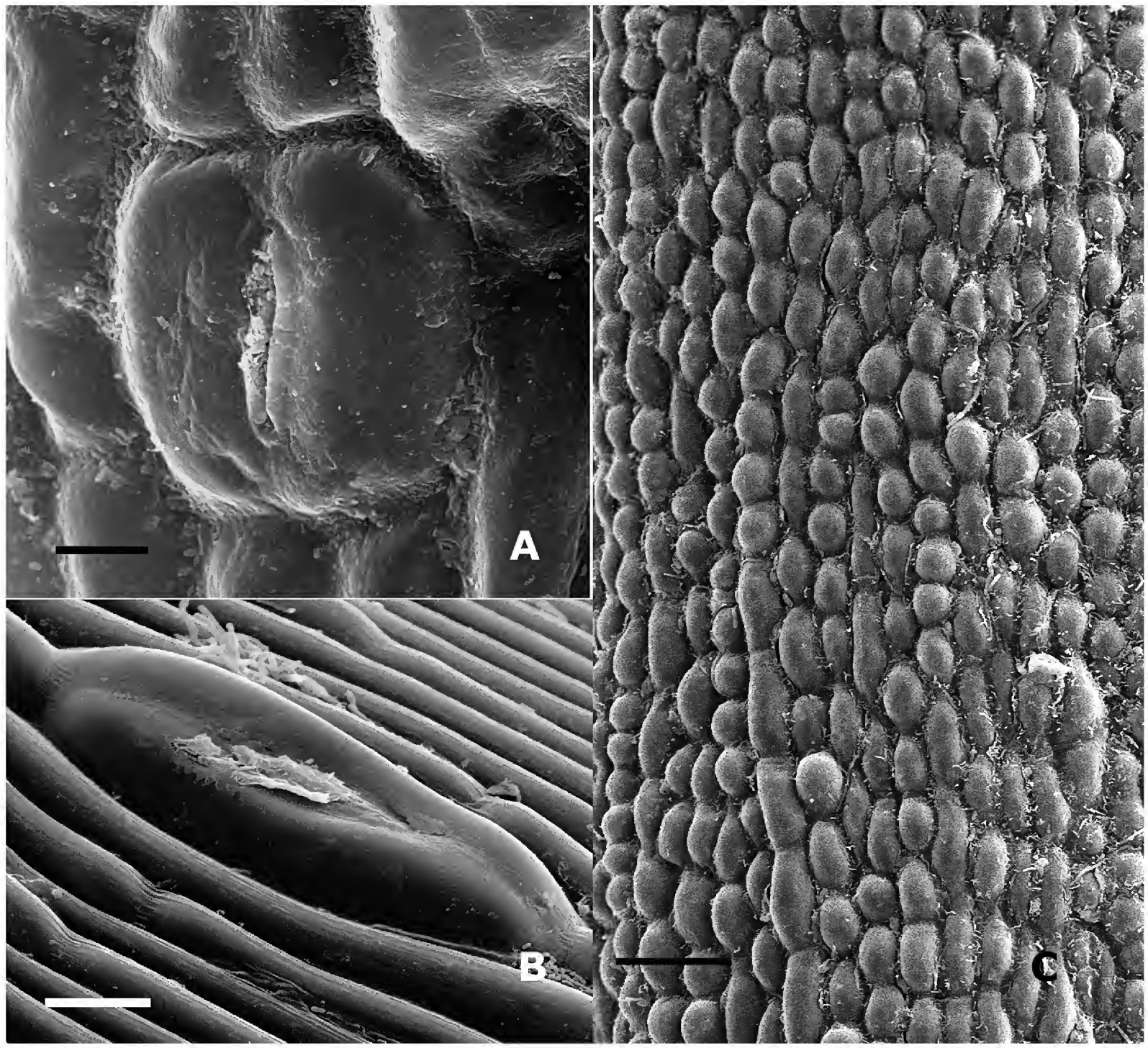

Figure 6. Scanning electron micrographs showing variations in the sporophyte epidermis of hornworts. —A. *Anthoceros macounii* M. Howe. Closed stoma with short guard cells. Bar = 10 μm. —B. *Folioceros fuciformis* (Mont.) D. C. Bharad. Side view of "typical" hornwort stoma. Bar = 10 μm. *A. Cairns s.n.* (CANB). —C. *Dendroceros crispatus* (Hook.) Nees. Sporophyte without stomata. Unlike most hornworts, epidermal cells in this species are not elongated. Bar = 40 μm. DCC 28 (CANB).

they each confirm the genetic isolation of this sample from other hornworts (Duff, unpublished).

The placement of *Leiosporoceros* among hornworts is highly variable in published classification schemes. Based solely on the possession of unusual small, monolete, smooth spores produced in isobilateral tetrads (Fig. 3A), Hässel de Menéndez (1986, 1988) segregated *Leiosporoceros* into an autonomous family and order (Table 1). In contrast, both Hasegawa and Schuster minimized the significance of tetrad and spore architecture, emphasizing that both tetrahedral and isobilateral spore tetrads have been found in single sporangia in ferns and liverworts (Hasegawa, 1988) and suggesting that a single gene mutation was likely responsible for the atypical isobilateral spore arrangement (Schuster, 1992). Schuster (1992) and Hasegawa (1986, 1988) stressed the commonality of yellow spores and gametophytic features such as a solid thallus for the placement of *Leiosporoceros* as a subgenus of *Phaeoceros* but cited a homology between the gametophytic outgrowths on *Leiosporoceros* and *Phaeoceros hirticalyx* (Steph.) J. Haseg. to support a close affinity between these taxa. On the other hand, Hyvönen and Piippo (1993) determined a sister relationship between *Leiosporoceros* and *Folioceros* based on the surface ornamentations of the involucre and pseudoelater characters, recognizing both taxa at the generic level.

Our studies of *Leiosporoceros* do not corroborate the conclusions of Hasegawa (1988), Schuster (1992), or Hyvönen and Piippo (1993); rather, care-

ful scrutiny of both generations has revealed remarkable new features that are unique to this hornwort. A cursory examination of *Leiosporoceros* gametophytes suggests some affinities with *Phaeoceros* or *Megaceros*. The thallus is fleshy, has external outgrowths, and lacks internal mucilage canals. Contrary to all other hornwort gametophytes in which *Nostoc* Vaucher occurs in discrete spherical colonies, this endosymbiont in *Leiosporoceros* occurs in branching strands that run longitudinally and are sequestered in the central thallus. Coupled with this novel arrangement of *Nostoc* is the apparent lack of ventral mucilage clefts, ubiquitous structures in other hornworts that enable *Nostoc* to enter and establish colonies in the lower thallus regions. Chloroplast ultrastructure is also peculiar in *Leiosporoceros* (Fig. 4A). At the light microscope level, a pyrenoid is suggested by the peripheral arrangement of starch. Closer examination reveals a central elaboration of photosynthetic membranes, not a pyrenoid in this plant. This arrangement is consistent with the existence of a carbon-concentrating mechanism typical of organisms with pyrenoids, including other hornworts (Smith & Griffith, 1996, 2000; Hansen et al., 2002).

In cross section, the sporophyte of *Leiosporoceros* is unmistakably a hornwort; however, novel features are evident (Fig. 5A). Unlike other hornwort taxa, the suture is highly differentiated and visible as a deep longitudinal groove in the sporophyte (Fig. 5A). The sporogenous layer contains numerous, widely separated spore tetrads and large, elongated and irregularly thickened pseudoelaters, all embedded in a mucilaginous matrix. Upon drying, the pseudoelaters often twist (Fig. 3A), giving an appearance that is reminiscent of spiraled elaters of *Megaceros* and *Dendroceros* (Fig. 3D, F). Stomata are common and occur scattered in the epidermis and are similar in size to those found in *Phaeoceros* and *Anthoceros* s.l. (Fig. 6A, B). Clearly, the complex of unique morphological traits in *Leiosporoceros* is supportive of the observed genetic isolation of this taxon among hornworts.

The second monophyletic hornwort assemblage includes *Anthoceros* and *Folioceros* (Fig. 1, Clade B). The observed sequence divergence between *Anthoceros* s.l. (including *Folioceros*) and the rest of the hornworts was unexpected. Prior treatments have typically accepted a sister relationship between *Anthoceros* and *Phaeoceros*, at least placing them in the same family (Fig. 1). Both taxa occupy similar habitats and share key morphological characteristics, including similar growth forms (Fig. 2A), chloroplast structure, with well-developed pyrenoids (Fig. 4C), and sporophyte anatomy (Fig. 5B). Despite this overall similarity in structure with *Phaeoceros*, *Anthoceros* and *Folioceros* exhibit diagnostic features that are best interpreted as synapomorphies. These include the possession of black or dark spores (in contrast to yellow spores at maturity in *Phaeoceros*) and the development of elaborate dorsal lamellae in some species and schizogenous mucilage cavities in the gametophyte. Similarly, antheridia of *Anthoceros* and *Folioceros* have a unique tiered architecture, an apical opening system, and they occur in large groups of up to 40. *Leiosporoceros* shares the large number of antheridia per chamber, while in all other taxa antheridia are solitary or occur in groups of up to six. Spore ornamentation (Fig. 3B) and wall ultrastructure also differentiate *Anthoceros* s.l. from other hornworts (Fig. 3A, C–F). Such similarities include the darkly pigmented spore wall, differential ornamentation patterns of the distal and proximal faces, and a well-defined trilete mark.

Our results are unambiguous (bootstrap 100%, see Fig. 1) in regard to the sister relationship between *Folioceros* and *Anthoceros*, and this association is reflected in most current classifications. However, the autonomy of *Folioceros* as a well-defined genus cannot be evaluated based on our analysis due to limited taxon sampling. Schuster (1992) and Hasegawa (1988, 1994) included *Folioceros* within *Anthoceros*, while Hyvönen and Piippo (1993) treated the genus as closely related to *Sphaerosporoceros*, *Leiosporoceros*, and *Anthoceros*. Only Hässel de Menéndez (1988) segregated *Folioceros* from other taxa, placing this genus in its own family and order. The rationale for this distinction is based solely on differences in spore and pseudoelater morphology between *Folioceros* and *Anthoceros*. Specifically, the elongated, thick-walled pseudoelaters of *Folioceros* are reddish brown in color and are decisively different from the short, thin-walled multicellular elaters that characterize *Anthoceros*. As noted by Hässel de Menéndez (1988) and Hyvönen and Piippo (1993), the structure of *Folioceros* pseudoelaters is reminiscent of that of *Leiosporoceros* (Fig. 3A), suggesting a sister relationship between these two taxa.

The remaining hornworts form a monophyletic group that comprises five well-supported assemblages (Fig. 1, Clades C–G). Four samples of *Phaeoceros carolinianus* (Michx.) Prosk. from different geographic locations show little sequence divergence (Fig. 1, Clade C), and this group plus *Notothylas* (Fig. 1, Clade D) is monophyletic. As discussed below, two other species of *Phaeoceros* sampled in our study nest within the *Megaceros* assemblage, supporting polyphyly of this genus. A

sister relationship between *Phaeoceros* and *Notothylas* was suggested only by Hässel de Menéndez (1988) who placed these two genera together in the family Notothyladaceae. Similarities between *Phaeoceros* and *Notothylas* include the possession of a pyrenoid in each chloroplast, an equatorial girdle in spores, and 2 to 4(to 6) antheridia per cavity. However, most modern classification schemes recognize the defining features of *Notothylas* as distinct enough to warrant elevation of this genus into a monotypic family (Singh, 2002).

Notothylas is the only hornwort taxon in which elongation of the sporophyte is determinate and spore production appears synchronized (Fig. 2C). As a result, from a phylogenetic perspective, *Notothylas* has received considerable attention. It has been suggested that the small, simple sporophytes of this genus represent a basal condition, with elaboration of sporophytes in the remaining, more derived taxa (Campbell, 1895; Mishler & Churchill, 1984; Graham, 1993; Hyvönen & Piippo, 1993; Hasegawa, 1994). Contrary to this conclusion, the *rbc*L data support the interpretation that sporophytes in *Notothylas* are not plesiomorphic but were derived through reduction and specialization, a speculation widely supported by previous workers (Lang, 1907; Bartlett, 1928; Proskauer, 1960; Renzaglia, 1978; Schofield, 1985; Schuster, 1992). A similar reduction series may have occurred independently in other genera, thus supporting the contention that *Notothylas* may be a polyphyletic hornwort assemblage (Lange, 1907; Proskauer, 1960).

Perhaps the least controversial phylogenetic relationship within the hornworts is the close affinity between *Dendroceros* (Fig. 1, Clade E) and *Megaceros* s.l. (Fig. 1, Clades F and G). These taxa share the possession of spiraled elaters (Fig. 3D, F), absence of stomata (Fig. 6C), and solitary antheridia (rarely 2 or 3), three seemingly derived characters. *Dendroceros* is further characterized by an epiphytic habit, thickened central midrib with perforated wings (Fig. 2B), large central pyrenoids in each plastid (Fig. 4D), and multicellular spores (Hasegawa, 1980; Renzaglia & Vaughn, 2000). Diagnostic features of *Megaceros* include unicellular spores with distal mammilla (Fig. 3E, F) and chloroplast ultrastructure (Fig. 4E) (Hasegawa, 1983; Valentine et al., 1986). *Megaceros* is distinguished among hornworts by the occurrence of small numerous plastids, especially in the internal cells of the thallus that lack pyrenoids (Fig. 4E) (Vaughn et al., 1992). Nonetheless, as noted by Proskauer (1953) and Hässel de Menéndez (1962) and reflected in the *rbc*L phylogeny, the distinction between *Dendroceros* and *Megaceros* is not absolute, especially in regard to growth form. Three *Dendroceros* species, from Old World and New World sites, form a well-supported monophyletic group (Fig. 1, Clade E) that is sister to a large and diverse *Megaceros* assemblage, which includes one species of *Dendroceros*, one of *Nothoceros*, and two species of *Phaeoceros* (Fig. 1, Clades F and G).

A primary dichotomy separates the *Megaceros* assemblage into two groups: the first with an assortment of taxa previously placed in four different genera (Fig. 1, Clade F) and the second contains a number of species morphologically similar to *Megaceros flagellaris* (Mitt.) Steph. (Fig. 1, Clade G). Characterized by a high degree of genetic and morphologic diversity, the seven taxa in the first *Megaceros* group (Fig. 1, Clade F) fall into two natural groups. Subclade F1 (Fig. 1) contains taxa from three named genera, *Megaceros*, *Nothoceros*, and *Dendroceros*. The genus *Nothoceros* was named as a subgenus of *Megaceros* by Schuster in 1987 and elevated to generic rank by Hasegawa in 1994. It included *Nothoceros giganteus* (Lehm. & Lindenb.) J. Haseg., a species formerly classified either in *Dendroceros* (Campbell, 1986; Proskauer, 1953; Hässel de Menéndez, 1962) or *Megaceros* (Hasegawa, 1988). Differentiation of the thallus into midrib and monostromatic wings led most workers to place this taxon in *Dendroceros*. Clear affinities with *Megaceros* are seen in the unicellular-mamillate spores and cells containing multiple chloroplasts without pyrenoids (Valentine et al., 1986). The growth form of *Dendroceros canaliculatus* Pagán likewise resembles that of *Dendroceros* (Pagán, 1942), albeit the midrib is not as sharply defined. Similarities in habit coupled with *Megaceros*-like spores led Hasegawa (1988) to propose the placement of *D. canaliculatus* and *D. fuegiensis* (Steph.) Hässel in *Megaceros* along with *M. giganteus* under the subgenus *Nothoceros* (sensu *Australoceros*, Hasegawa, 1988). *Dendroceros canaliculatus* was only previously known from Luquillo Mountains, Puerto Rico (Pagán, 1942), but recent Central American collections, including the one in this study, show high variability in thallus morphology, with some individuals resembling the growth form of the North American *M. aenigmaticus*, while others are similar to those from Puerto Rico (Villarreal & Salazar Allen, unpublished).

The remaining members of this morphologically diverse clade (Fig. 1, Subclade F1) are the Neotropical *M. vincentianus* (Lehm. & Lindenb.) Campb., one undescribed *Megaceros* species, and *Megaceros aenigmaticus* Schust., the only representative of the genus in North America. Unlike most *Megaceros*, this latter species is dioicous, with male

and female plants separated into different watersheds (Renzaglia & McFarland, 1999). Consequently, sporophytes are not known in *M. aenigmaticus*, obscuring the precise placement of this species within the genus. A relationship between *M. aenigmaticus* and *M. vincentianus* was speculated by Schuster in 1992. As the only species of *Megaceros* recognized in the Neotropics, *M. vincentianus* is a highly variable taxon with unclear status (Burr, 1970; Vaughn et al., 1992; Gradstein et al., 2001; Villarreal & Salazar Allen, 2002). Morphological studies of *M. vincentianus* have identified substantial diversity among populations, thus suggesting that more than one species may be at hand (Villarreal & Salazar Allen, 2002).

The results presented here do not support the autonomy of *Nothoceros* and the placement of *D. canaliculatus* in the genus *Dendroceros*; these taxa are more appropriately placed in *Megaceros*. Subgeneric status of a revised *Nothoceros* assemblage that includes all members of the F1 subclade may be in line but determination of this taxonomic revision will await further sampling. A further conclusion supported by our analysis is that the thickened midrib and monostromatic wings in *Nothoceros giganteus* and *D. canaliculatus* are not homologous to that in *Dendroceros* but were likely the result of convergent evolution.

Two samples of *Phaeoceros* from New Zealand, (Fig. 1, Subclade F2), *P. coriaceus* (Steph.) E. O. Campbell and *P. hirticalyx*, form an assemblage that is sister to the F1 *Megaceros* clade. In addition, sequence data from mitochondrial (*nad*2 and *nad*5) and nuclear (18S SSU rDNA) genomes corroborate the genetic similarity of these species with *Megaceros* (Duff, unpublished). This placement is problematic in light of the presence of yellow spores, stomata, and the lack of helical thickenings in pseudoelaters, features that identify these species as members of the genus *Phaeoceros* (Proskauer, 1951). However, further examination reveals that the chloroplast ultrastructure and monandrous androecia in both plants indicate a relationship with *Megaceros*. As is typical of *Megaceros* species, the chloroplasts of *P. coriaceus* and *P. hirticalyx* often number more than one per cell and lack a pyrenoid (Vaughn et al., 1992). These results support the interpretation that within the *Megaceros* assemblage loss of the pyrenoid and increase in plastid number preceded the loss of stomata and the production of spiraled pseudoelaters. Testing this hypothesis will require sampling from additional *Phaeoceros* and *Megaceros* species. Minimally, our results call into question traditional views on diagnostic morphological traits that delimit generic boundaries in hornworts (Cargill et al., submitted), as was noted nearly a century ago by Bartlett (1928). Similarities in habit, overlapping habitats, and shared morphological features serve to confound distinction between *Phaeoceros* and *Megaceros*.

The remainder of the species of *Megaceros* (Fig. 1, Clade G) form an Australasian group that consists of species morphologically similar to *Megaceros flagellaris*. The low level of genetic diversity within this group supports the suggestion that many of the Asian and Austral species of *Megaceros* may be synonymized either under *M. flagellaris* (Hasegawa, 1983) or under *M. pellucidus* (Colenso) E. A. Hodgs. (Glenny, 1998). Only *M. denticulatus* (Lehm.) Steph., a dioicous species from New Zealand, can easily be distinguished genetically from the other members of this clade. Nonetheless, *rbc*L data have not normally been used to answer species or population level questions, and recent studies of spore ornamentation within the Australasian populations suggest a number of species may exist within the group (Cargill & Vella, unpublished).

MORPHOLOGICAL INFERENCES

Reappraisal of morphological diversity vis à vis this novel hypothesis on hornwort interrelationships leads to intriguing new evolutionary inferences. Below we re-examine the evolution of chloroplast and stomata, two controversial morphological features of hornworts.

CHLOROPLAST EVOLUTION

The hornwort chloroplast shares features with green algal chloroplasts and shows no parallel in any other embryophyte group. All have unique intramembrane systems including the lack of grana end walls (Fig. 4C) and elaborate channel or tubular thylakoid networks that enclose internal space (Vaughn et al., 1992). Pyrenoids are large central regions that typically contain lens-shaped subunits traversed by thylakoids or grana (Fig. 4B, C). Pyrenoids are lacking in *Leiosporoceros* (Fig. 4A), *Phaeoceros coriaceous*, *P. hirticalyx*, and *Megaceros* (Fig. 4E), with zonation within the organelle evident in the first three and less so in the latter. Species of *Notothylas*, *Anthoceros*, and *Folioceros* have also been reported to lack pyrenoids (Ashtana & Srivastava, 1991; Vaughn et al., 1992; Singh, 2002). An evolutionary inference based on the *rbc*L hypothesis is that a solitary chloroplast with a pyrenoid is plesiomorphic in hornworts. In *Leiosporoceros* the plastid remained solitary but lost the pyrenoid, the remnant of which is a compartmentalized organelle with peripherally aggregated

starch and centralized grana (Fig. 4A). A secondary loss of the pyrenoid occurred in the *Megaceros* s.l. lineage with compartmentalization partially retained (pyrenoid-like area of some *Megaceros*) or entirely lost as in most taxa (Fig. 4E). Pyrenoid loss in *Megaceros* was accompanied by an increase in number and decrease in size of the chloroplast. As exemplified by *Dendroceros*, with irregularly shaped pyrenoid subunits (Fig. 4A), modifications of pyrenoid structure are specific to and diagnostic of each lineage. The loss of pyrenoids in more than one line of hornworts finds parallels with the multiple losses (and gains) of this region in algal lineages (Hoham et al., 2002; Nozaki et al., 2002). Because hornworts presumably retained the pyrenoid from an algal ancestor and are the only land plants that have done so, continued studies of the structure and physiology of a wide range of hornwort taxa have the potential to clarify evolutionary modifications that occurred in this organelle, not only within hornworts but among all land dwelling plants.

EVOLUTION OF STOMATA

Stomata are a significant innovation in the diversification of land plants, and their presence in hornworts has been alternately viewed as a shared trait with mosses and tracheophytes or an independent acquisition that accompanied sporophyte elaboration within the hornworts (Mishler & Churchill, 1984; Kenrick & Crane, 1997; Renzaglia et al., 2000). It has been argued that the mucilage clefts on the ventral side of the gametophyte are homologous to sporophytic stomata (Proskauer, 1951; Schuster, 1992), thus providing the only example in extant plants where stomata are found in both life cycle phases. The lack of mucilage clefts in *Leiosporoceros*, as well as the very specialized function of these structures as a site of *Nostoc* entrance in all other hornworts, suggest that gametophytic mucilage clefts evolved after hornwort diversification and are not homologous to sporophytic stomata. Moreover, the abundance of stomata on the sporophyte of *Leiosporoceros* supports the interpretation that stomata are plesiomorphic in hornworts and that they are homologous to those in other plant groups. One caveat in regard to this speculation is that although hornwort stomata function in gas exchange, they do not appear to undergo diurnal changes, i.e., once open, they never close (Paton & Pierce, 1958; Lucas & Renzaglia, unpublished). A clear case of homoplasy is the loss of stomata in at least two, possibly three, hornwort lineages: *Notothylas*, *Dendroceros*, and *Megaceros*. Because sporophytes of *Notothylas*, especially, and *Dendroceros*, less so, undergo most of their development within the protective involucre where gas exchange with the environment is inhibited, it is not surprising that stomata were lost in these taxa. It may be argued that the lack of stomata in *Megaceros* is related to the highly elongated involucre that surrounds the sporophytes during tissue differentiation and/or the occurrence of members of this genus in wet or periodically flooded habitats. The presence of stomata on the sporophytes of the terrestrial taxa *P. coriaceus* and *P. hirticalyx*, which have short involucres, supports this contention. It is possible that genetic potential to produce stomata was not lost in *Notothylas*, *Dendroceros*, and *Megaceros*, but rather the production of these structures simply may be suppressed by the enclosure of the sporophyte within the involucre during critical developmental stages. This phenomenon, which may be likened to the lack of stomata in aquatic angiosperms, would explain the occurrence of stomata within *P. hirticalyx* and *P. coriaceous*. Rigorous empirical testing of this hypothesis is required.

Summary and Conclusions

The discussion above serves to illustrate the chaotic nature of hornwort taxonomy and classification. Although hornworts are easily identified by the unique structure of their sporophyte and chloroplast, considerable diversity exists in a range of morphological and ultrastructural traits in these plants. Intermediates in many of these characteristics obscure demarcation between genera and species. Highly divergent views exist on which morphological features are taxonomically useful. Assessments of morphological and genetic diversity among multiple species and populations across a wide geographic range are essential to further resolve relationships and elucidate taxonomically informative features of these plants. This will require inclusion of the three hornwort taxa not included in our study, namely *Mesoceros*, *Hattorioceros*, and *Sphaerosporoceros*. Each of these plants poses anomalies that extend the limits of morphological diversity represented in the 20 samples included here. As did *Leiosporoceros*, each has the potential to dramatically influence at least some of the relationships described above.

Morphological extremes exhibited by hornwort genera or species likely reflect adaptations to the different environments they inhabit. *Dendroceros* is epiphytic, *Megaceros* often grows in sites that are periodically inundated, and *Notothylas* occurs in highly disturbed habitats with periodic flooding.

Conversely, similar morphological features among *Phaeoceros*, *Leiosporoceros*, and *Anthoceros* may be a function of their occurrence on exposed soil, a habitat found across long evolutionary time periods, which may represent the prototypic hornwort environment. Based on the *rbc*L analysis, it could be argued that the habit and morphology of extant members of *Anthoceros*, *Leiosporoceros*, and *Phaeoceros* represent the ancestral conditions of hornworts, a condition that may have undergone few changes over the hundreds of millions of years since their diversification. Subsequently, radiation occurring into more specialized habitats resulted in specializations in growth form, sporophyte structure, and chloroplast condition.

Only now are we beginning to understand and document diversity within the ancient and intriguing hornwort lineage. Enumerating this diversity has required examination of features and structures at levels heretofore unexplored. Our *rbc*L analysis is a critical first step in clarifying interrelationships among hornwort taxa as well as in elucidating the directionality of morphological change. For the most part, the relationships supported by this phylogeny are highly intuitive, and further examination of morphological characters provides exciting new insights on hornwort evolution. As with any new revolutionary scientific hypothesis, rigorous testing is in order. Molecular studies such as this, coupled with detailed morphological investigations, will continue to revolutionize traditional interpretations of interrelationships and character evolution within hornwort and among basal embryophytes. Such data are essential to unravel the evolutionary history of terrestrial plants.

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A MOLECULAR PHYLOGENY OF LEAFY LIVERWORTS (JUNGERMANNIIDAE: MARCHANTIOPHYTA)[1]

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Abstract

Nucleotide sequences from two nuclear (26S and 18S), three mitochondrial (*nad*1, *trn*S, *nad*5), and seven chloroplast loci (*atp*B, *rbc*L, *psb*T, *trn*G, *trn*L, *psb*A, *rps*4) were used to reconstruct phylogenetic relationships among major clades of liverworts based on a sample of 20 liverworts and three moss outgroups. A more detailed analysis of phylogenetic relationships among the leafy liverworts utilized four loci (26S, *psb*A, *rps*4, *nad*5) and a sample of 81 liverworts, two mosses, and one hornwort. Maximum parsimony, maximum likelihood, and Bayesian analyses were conducted on each data set in order to establish a robust hypothesis for phylogenetic relationships among the leafy liverworts. Results of these analyses suggest that (1) the phylogeny departs substantially from arrangements predicted by two recent classification schemes; (2) the leafy liverworts are composed of two large clades that represent an early dichotomy; (3) *Pleurozia* is more closely related to the simple thalloids than to leafy liverworts; (4) the leafy liverworts previously held as most advanced are not a crown group; (5) the liverworts previously held as most primitive are dispersed throughout the phylogeny; (6) simple thalloid characteristics appear to represent liverwort plesiomorphies; and (7) *Haplomitrium* may be sister to all other liverworts or sister to the Jungermanniopsida.

Key words: Bayesian analysis, Jungermanniidae, Jungermanniopsida, leafy liverworts, molecular systematics, morphological evolution, phylogeny.

Liverworts (Marchantiophyta Stotler & Stotl.-Crand.) represent one of the few remaining land plant divisions for which the broad outline of phylogenetic structure has barely been investigated using molecular tools. Comprised of approximately 8000 species (Schuster, 1984), the liverworts are an ancient lineage of embryophytes that display a remarkable diversity of morphologies. Characteristics, such as the lack of sporophytic stomata and IAA (auxin) conjugation, the absence of certain mitochondrial introns and mitochondrial editing, and analyses of nucleotide sequence data have led some to argue that liverworts are the earliest lineage of embryophytes, although this remains controversial (Mishler & Churchill, 1984; Mishler et al., 1994; Hedderson et al., 1996; Lewis et al., 1997; Garbary & Renzaglia, 1998; Hedderson et al., 1998; Qiu et al., 1998; Beckert et al., 1999; Nishiyama & Kato, 1999; Steinhauser et al., 1999; Nickrent et al., 2000; Renzaglia et al., 2000; Pruchner et al., 2001; Kugita et al., 2003; Davis et al., in review).

Liverworts are Monophyletic

The liverworts are defined by a unique set of characteristics that separate them from all other embryophytes: (1) the presence of unicellular elaters in the capsule, derived from the division of a sporangial cell into one sporocyte initial and one elater initial, with the former undergoing mitotic divisions to produce an 8 : 1 or 4 : 1 spore : elater ratio; (2) complete maturation of the spores prior to the elongation of the sporophyte seta; (3) a non-photosynthetic (generally) ephemeral sporophyte; and (4) the presence of membrane-bound oil bodies (Crandall-Stotler & Stotler, 2000). These features, along with DNA sequence data, support the hypothesis that liverworts form a monophyletic group (Mishler & Churchill, 1984; Mishler et al., 1992, 1994; Garbary et al., 1993; Hedderson et al., 1996; Lewis et al., 1997; Garbary & Renzaglia, 1998; Beckert et al., 1999; Nishiyama & Kato, 1999; Crandall-Stotler & Stotler, 2000; Renzaglia et al.,

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2000). These studies have generally supported two mutually monophyletic lineages of liverworts, one containing taxa with so-called complex thalloid gametophytes (Marchantiopsida Stotler & Stotl.-Crand.) and another containing those with leafy and simple thalloid gametophytes (Jungermanniopsida Stotler & Stotl.-Crand.). Beyond verification of liverwort monophyly and identification of this dichotomy, which agrees with modern classifications (Schuster, 1984; Crandall-Stotler & Stotler, 2000), surprisingly little molecular-based phylogenetic research focusing on liverwort relationships has been reported.

Liverwort Phylogeny

The Marchantiopsida consist of 31 genera and approximately 485 species (Schofield, 1985). Some of their defining characteristics include a typically multistratose thallus, which is usually differentiated into tissue layers with epidermal air pores (except *Sphaerocarpus* and its relatives), complex gametangiophores (except in *Riccia* and its relatives), four primary androgonial cells, unistratose capsule walls, and spore mother cells which are unlobed (Crandall-Stotler & Stotler, 2000). Four phylogenetic studies specifically addressing relationships within this group have been published (Boisselier-Dubayle et al., 1997; Bischler, 1998; Long et al., 2000; Wheeler, 2000). These studies represent considerable progress toward understanding phylogeny and character evolution within the Marchantiopsida.

The Jungermanniopsida contain at least 85% of the taxon diversity in hepatics (Schuster, 1984), with approximately 300 genera and 7500 species (Schofield, 1985). They are typified by a unistratose gametophyte (rarely multistratose, and then lacking internal differentiation and epidermal air pores), two or morestratose capsule walls (unistatose in *Haplomitrium*), two primary androgonial cells (except one in *Haplomitrium*), and lobed sporocytes (Crandall-Stotler & Stotler, 2000). They are classified into two main groups: the leafy liverworts and the simple thalloid liverworts. Most diverse is the leafy group (approximately 280 genera, 7000 species; Schofield, 1985). Leafy liverworts (Jungermanniidae Engl. emend. Stotler & Stotl.-Crand.) are differentiated from simple thalloid liverworts (Metzgeriidae Barthol.-Began) by a tetrahedral apical cell with three cutting faces (except *Pleurozia*), leaves that are formed from two initials, capsules that always dehisce into four valves, and acrogyny, wherein the archegonia terminate the main stem (Crandall-Stotler & Stotler, 2000). In contrast, in the simple thalloids, apical cell shape and the number of capsule valves are variable, in taxa with leaf-like thallus projections, these leaves are formed from a single initial, and the stems can be acrogynous or anacrogynous (archegonia produced laterally). The wide variation in simple thalloid gametophyte morphology sheds doubt on their status as a natural group, and published phylogenetic analyses indicate that it is paraphyletic (Lewis et al., 1997; Crandall-Stotler & Stotler, 2000). The three published phylogenetic studies on the Jungermanniidae suffer from lack of resolution and support due to limited taxon sampling and/or few sampled loci; its monophyly is inconsistently supported, and no clear consensus of phylogenetic relationships within this group has emerged (Lewis et al., 1997; Crandall-Stotler & Stotler, 2000; Stech & Frey, 2001).

Morphological Evolution

Hypotheses regarding relationships among the three groups, complex thalloid, simple thalloid, and leafy liverworts, have been controversial (see Schuster, 1984, for a comprehensive review). Historically, this controversy arose due to disagreement about the direction in which morphological evolution should be read. Earlier classifications held that the simple thalloid taxa were most primitive and that the complex thalloid and leafy taxa were derived from them, with the leafy taxa being the most derived (Endlicher, 1841). Later classifications reversed the order, favoring instead the hypothesis that the leafy taxa were most primitive, and the complex thalloid taxa were the most derived (Gottsche et al., 1844–1847; Verdoorn, 1932; Evans, 1939; this arrangement is also consistent with the system originally proposed by Linnaeus in 1753). These later classifications were based on the belief that the massive sporophytes found in leafy liverworts, such as *Lepicolea,* were primitive, while the more reduced sporophytes found in the complex thalloids were more advanced (a reductionist viewpoint). Both sets of arguments were rejected by Schuster (1984), who proposed that the ancestral liverwort was likely upright and leafless, though without empirical evidence from fossils or phylogenetic analyses of extant taxa. These ancestral liverworts then evolved, he proposed, into two unique lineages, the marchantioid and the jungermannioid; an event he termed the initial dichotomy (Schuster, 1984). In other words, Schuster speculated that the jungermannioids and marchantioids are sister groups, rather than one being nested within the other.

Classification and Evolutionary Hypotheses for the Leafy Liverworts

The phylogenetic position of the genus *Haplomitrium* is potentially important for interpreting morphological evolution in the leafy liverworts. *Haplomitrium* is a leafy taxon, primarily of Indian and Southeast Asian distribution, with problematical species delimitations (Schuster, 2000). A few species occur in the Southern Hemisphere, and one species is widely distributed, but locally rare, in the Northern Hemisphere (*H. hookeri* (Sm.) Nees). The genus has a suite of seemingly contradictory characteristics. In overall appearance, it looks somewhat like a moss because of its upright habit and isophyllous leaves; its capsule wall is unistratose, as in the marchantioid liverworts, but the cell walls have unique secondary thickenings; its archegonia are naked and scattered randomly on the gametophyte; its long, filamentous elaters are most like those found in some simple thalloids; its seta is chlorophyllose when young, and the gametophyte lacks rhizoids. The classification of Schuster (1966, 1984) placed the genus at the beginning of his classification in the order Calobryales (the order also included the genus *Takakia,* which has since been shown to be a moss, not a liverwort (Renzaglia et al., 1997)). The placement of this taxon at the beginning of Schuster's classification reflected the idea that the suite of morphological traits characterizing *Haplomitrium* represents the putative ancestral condition in the jungermannioids. Thus, the upright, isophyllous leafy liverworts are placed at the beginning of the order Jungermanniales (e.g., *Herbertus*). Prostrate taxa with dorsiventral gametophytes and pronounced anisophylly (with the underleaves clearly differentiated from the lateral leaves) follow as more derived. Among these dorsiventral groups, taxa with lateral leaves that become folded and differentiated into lobes and lobules, where the lobule may be flap-like or form a water-sac (i.e., complicate-bilobed or lobulate), are listed as the most advanced (e.g., *Lejeunea, Radula, Frullania*). The subsequent classification of Crandall-Stotler and Stotler (2000) largely followed the classification of Schuster (1984); however, *Haplomitrium* was placed within the simple thalloids rather than in a unique order at the beginning of the classification. This was based on observations that, despite its leafy gametophyte, *Haplomitrium* shares many characteristics with the simple thalloids, including leaf development from a single initial.

Other notable characters that have been interpreted as primitive in the leafy liverworts include massive sporophytes and elaborated protective structures around the developing embryo (e.g., a coelocaule). This interpretation reflects the reductionist viewpoints of Gottsche et al. (1844–1847), Verdoorn (1932), and Evans (1939). Fasciculate rhizoids and multiple-lobed leaves are also interpreted as primitive in modern classifications (Schuster, 1984). In addition, leafy liverworts display a wide range of morphological diversity in leaf shape and insertion, branching patterns, types and shapes of protective tissue surrounding the archegonia, arrangement and number of seta cells, and sporangium and spore ornamentation. Such characters have been used to create classifications and to make inferences about morphological evolution (Schuster, 1966, 1984). These hypotheses have thus far remained untested using phylogenetic methods.

Study Objectives

The primary goals of this study were: (1) to develop a well-supported phylogenetic framework of leafy liverwort phylogeny within which evolutionary hypotheses about morphology can be tested; (2) to determine whether natural groups as outlined in the classification schemes of Schuster (1984) and Crandall-Stotler and Stotler (2000) are supported by the phylogeny; and (3) to evaluate the phylogenetic position of *Haplomitrium.* Toward these goals, a data matrix was constructed for 84 taxa and four loci. Analyses of these data showed that while clades could be resolved with four genes, there was insufficient resolution of relationships among major clades. In order to establish a robust backbone for the phylogeny, an additional data set was constructed with 12 loci for a reduced sample of taxa representing major clades resolved in the first analysis.

Materials and Methods

Sampling for the four-gene, 84-taxon analyses: full matrix

Eighty-four taxa, including two mosses, a hornwort, and 81 liverworts, were chosen as exemplars (Table 1). Taxa were chosen to represent as many families of leafy liverworts as possible (30 of 42) following the classification of Crandall-Stotler and Stotler (2000). Sequence data for 26S nrDNA, *nad*5 mtDNA, and *psb*A, and *rps*4 cpDNA were obtained from GenBank or generated for this study (289 novel sequences). For five species, when *nad*5 sequences were not successfully obtained in the lab and were unavailable on GenBank, GenBank *nad*5 sequences from closely related species were substituted: *Andreaea rupestris* Hedw. for *Andreaea wil-*

Table 1. Voucher information and GenBank accession numbers for specimens in the full data matrix. Non-novel sequences are marked with an asterisk (*). Dashes (—) indicate that no sequence was obtained.

Species	Collection	GenBank accession No.			
		*rps*4	*psb*A	26S	*nad*5
INGROUP TAXA					
Acromastigum exile (Lindenb.) A. Evans	Kenya, *Chua-Petiot Mb28* (NY)	AY608041	AY607919	AY608192	—
Adelanthus lindenbergianus (Lehm.) Mitt.	Argentina, *Drehwald 970083* (NY)	AY608042	AY607920	AY608193	AY608262
Aneura pinguis (L.) Dumort.	Russia, *Bakalin & Bakalin* [acc. 1][1] (NY)	AY608043	AY607921	AY608195	AY608263
Anthelia julacea (L.) Dumort.	Canada, *Schofield & Schofield 102042* (DUKE)	AY608044	AY607922	AY608196	—
Apometzgeria frontipilis (Lindb.) Kuwah. & J. J. Engel	Argentina, *Drehwald 970084* (NY)	AY608045	AY607924	AY608197	AY608264
Ascidiota blepharophylla subsp. *alaskana* Steere & R. M. Schust.	U.S.A., *Lewis 514* (F)	AY608046	AY607925	AY608198	AY608265
Balantiopsis diplophylla (Hook. f. & Taylor) Mitt.	Australia, *Streimann 59554* (NY)	AY608047	AY607926	AY608199	AY608266
Bazzania sp. indet.	Jamaica, *Davis 146* (DUKE)	AY608048	AY607927	AY608200	AY608267
Blasia pusilla L.	U.S.A., *Hicks & Davison 2392* (DUKE)	AY608049	AY607928	AY608201	AY608268
Blepharostoma trichophyllum (L.) Dumort.	U.S.A., *Schofield 111132* (DUKE)	AY608050	AY607929	—	—
Bryopteris filicina (Sw.) Nees	Bolivia, *Churchill, Magombo & Price 19855* (NY)	AY608051	AY607930	AY608202	AY608269
Calypogeia muelleriana (Schiffn.) K. Müll.	U.S.A., *Davis 130* (DUKE)	AY608052	AY607931	AY608203	—
Cephalozia catenulata (Huebener) Lindb.	U.S.A., *Anderson 26795* (DUKE)	AY608053	AY607932	—	AY608270
Cephaloziella hirta (Steph.) R. M. Schust.	Australia, *Streimann 59793* (NY)	AY608054	AY607933	AY608204	AY608271
Ceratolejeunea coarina (Gottsche) Steph.	Brazil, *Zartman 1235.1* (DUKE)	AY608055	AY607934	AY608205	AY608272
Chiloscyphus appalachianus (R. M. Schust.) J. J. Engel & R. M. Schust.	U.S.A., *Davison & Hicks 2934* (DUKE)	AY608056	AY607935	AY608206	AY608273
Cryptothallus mirabilis Malmb.	Belgium, Oct. 2001, *Boudier* (CONN)	AY608057	AY607936	AY608207	AY608274
Cyclolejeunea convexistipa (Lehm. & Lindenb.) A. Evans	Brazil, *Zartman 1232.1* (DUKE)	AY608058	AY607937	AY608208	AY608275
Diplophyllum albicans (L.) Dumort.	France, *Vitt w/Frahm 36705* (F)	AY608060	AY607939	AY608210	AY608277
Dumortiera hirsuta (Sw.) Nees	U.S.A., *Goffinet 6462* (DUKE)	AY608061	AY607940	AY608211	AY608278
Fossombronia pusilla (L.) Dumort.	Sargent living culture collection, Berkeley (UC)	AY608062	AY607941	AY608212	AY608279
Frullania cf. *madothecoides* Spruce	Ecuador, *Davis 295* (DUKE)	—	AY607942	AY608213	AY608280
Gackstroemia weindorferi (Herzog) Grolle	Australia, *Streimann 58531* (NY)	AY608063	AY607943	AY608214	AY608281
Goebeliella cornigera (Mitt.) Steph.	New Zealand, *von Konrat 2000/197* (F)	AY608064	AY607944	—	—

Table 1. Continued.

Species	Collection	GenBank accession No.			
		*rps*4	*psb*A	26S	*nad*5
Gymnomitrion concinnatum (Lightf.) Corda	Canada, *Davis 424* (DUKE)	AY608065	AY607945	AY608215	—
Haplomitrium blumei (Nees) R. M. Schust.	Guadeloupe, *Goffinet 1904* (CONN)	AY608066	AY607946	AY608216	AY608282
Haplomitrium gibbsiae (Steph.) R. M. Schust.	New Zealand, *Engel & von Konrat 24919* (F)	AY608067	AY607947	—	AY608283
Haplomitrium hookeri (Sm.) Nees	U.S.A., *Schofield 95224* (DUKE)	AY608068	*AY312904	*AY330437	AY608284
Harpanthus scutatus (F. Weber & D. Mohr) Spruce	U.S.A., *Risk, Dourson & Ratliff 10341* (DUKE)	AY608069	AY607948	AY608217	—
Herbertus sakurai (Warnst.) S. Hatt.	Sargent living culture collection, Berkeley (UC)	AY608070	AY607949	AY608218	AY608285
Herbertus subdentatus (Steph.) Fulford	Ecuador, *Davis 342* (DUKE)	AY608071	AY607950	AY608219	AY608286
Herzogobryum teres (Carrington & Pearson) Grolle	New Zealand, *Braggins 92/102* (F)	AY608072	AY607951	AY608220	—
Isotachis lyallii (R. M. Schust.) Mitt.	New Zealand, *Engel 21825* (F)	AY608073	AY607952	AY608221	AY608287
Jamesoniella autumnalis (DC.) Steph.		—	—	—	*AJ000700
Jamesoniella colorata (Lehm.) Steph.	New Zealand, *Engel 21964* (F)	AY608074	AY607953	AY608222	—
Jubula pennsylvanica (Steph.) A. Evans	U.S.A., *Risk 11005* (DUKE)	AY608075	AY607954	AY608223	AY608288
Jubulopsis novae-zelandiae (E. A. Hodgs. & S. W. Arnell) R. M. Schust.	New Zealand, *von Konrat 99/Feb. #16* (F)	AY608076	AY607955	AY608224	AY608289
Jungermannia cordifolia subsp. *exsertifolia* (Steph.) Amak.	Canada, *Davis 431* (DUKE)	AY608077	AY607956	AY608225	—
Jungermannia crenuliformis Austin	U.S.A., *Risk 11014* (DUKE)	AY608708	AY607957	AY608226	AY608290
Lejeunea cavifolia (Ehrh.) Linb.		—	—	—	*AJ000701
Lejeunea cladogyna A. Evans	U.S.A., *Davis 63* (DUKE)	AY608079	AY607958	—	—
Lepicolea ochroleuca (L. f. ex Spreng.) Spruce	Chile, *Drehwald 970188* (NY)	AY608080	AY607959	—	—
Lepicolea rara (Steph.) Grolle	Papua New Guinea, *Norris 66575* (DUKE)	AY608081	AY607960	AY608227	AY608291
Lepidolaena sp. indet.	New Zealand, *Engel 23052* (F)	AY608082	AY607961	AY608228	AY608292
Lepidozia reptans (L.) Dumort.	Sargent living culture collection, Berkeley (UC)	AY608083	AY607962	AY608229	AY608293
Lethocolea glossyphylla (Spruce) Grolle	Ecuador, *Davis 259*	AY608084	AY607963	AY608230	AY608294
Lophocolea bidentata (L.) Dumort.	U.S.A., *Davis 393* (DUKE)	AY608085	AY607964	AY608231	AY608295
Lophozia sp. indet.	U.S.A., *Davis 432* (DUKE)	AY608086	AY607965	AY608232	AY608296
Marchantia polymorpha L.		*NC_001319	*NC_001319	*AF226020	*M68929
Marsupella aquatica (Lindenb.) Schiffn.	France, *Frahm 90/589* (F)	AY608087	AY607966	—	AY608297
Marsupidium latifolium R. M. Schust.	Costa Rica, *Dauphin 2920* (NY)	AY608088	AY607967	AY608233	AY608298

Table 1. Continued.

Species	Collection	GenBank accession No.			
		*rps*4	*psb*A	26S	*nad*5
Mastigophora diclados (Brid. ex F. Weber) Nees	Tanzania, *Pocs 89119/V* (F)	AY608089	AY607968	—	—
Metzgeria sp. indet.	Ecuador, *Davis 361* (DUKE)	AY608090	AY607969	AY608234	—
Monoclea sp. indet.	Mexico, *Shaw 10151* (DUKE)	AY608091	AY607970	AY608235	AY608299
Nardia scalaris S. Gray	U.S.A., *Davis 438* (DUKE)	AY608092	AY607971	AY608236	—
Neotrichocolea bisseti (Mitt.) S. Hatt.	Japan, *Inoue* (F)	AY608093	AY607972	AY608237	AY608300
Nowellia curvifolia (Dicks.) Mitt.	U.S.A., *Risk & Gross 12220* (DUKE)	AY608094	AY607973	AY608238	AY608301
Odontolejeunea lunulata (F. Weber) Schiffn.	Brazil, *Zartman 1235.2* (DUKE)	AY608095	AY607974	AY608239	AY608302
Odontoschisma denudatum (Nees) Dumort.	U.S.A., *Horn 1809* (DUKE)	AY608096	AY607975	AY608240	AY608303
Palavicinia lyellii (Hook.) Gray	U.S.A., Feb. 1997, *Shaw* (DUKE)	AY608097	AY607976	AY608241	AY608304
Pellia epiphylla (L.) Corda	U.S.A., *Risk & Gross 12231* (DUKE)	*AY330479	*AY312912	AY608242	AY608305
Petalophyllum ralfsii (Wilson) Nees & Gottsche	Sargent living culture collection, Berkeley (UC)	AY608098	AY607977	AY608243	AY608306
Plagiochila asplenioides (L.) Dumort.		—	—	—	*AJ000704
Plagiochila austinii A. Evans	U.S.A., *Risk 10849* (DUKE)	AY608099	AY607978	AY608244	—
Pleurozia purpurea Lindb.	U.S.A., *Schofield 102815* (DUKE)	AY608100	AY607979	—	AY608307
Porella pinnata L.	U.S.A., *Goffinet 4744* (DUKE)	AY608101	AY607980	AY608245	AY608308
Preissia quadrata (Scop.) Nees	U.S.A., *Schofield 105579* (DUKE)	AY608102	AY607981	AY608246	AY608309
Ptilidium ciliare (L.) Hampe	Canada, *Schofield 103486* (DUKE)	AY608103	AY607982	AY608247	AY608310
Radula complanata (L.) Dumort.	Russia, *Bakalin* [acc. 8][1] (NY)	AY608104	AY607983	—	AY608311
Radula perrottetii Gottsche ex Steph.	Japan, *Mizutani 15030* (F)	AY608105	AY607984	AY608248	AY608312
Riccardia sp. indet.	Jamaica, *Davis 151* (DUKE)	AY608106	AY607985	AY608249	AY608313
Riccia fluitans L.	U.S.A., *Davis 103* (DUKE)	AY608107	AY607986	*AF226005	—
Scapania nemorosa (L.) Dumort.	U.S.A., *Davis 124* (DUKE)	AY608108	AY607987	AY608250	AY608314
Schistochila lehmanniana (Lehm. & Lindenb.) Carrington & Pearson	Australia, *Streimann 58554* (NY)	AY608109	AY607988	AY608251	—
Sphaerocarpos donnelli Austin		—	—	—	*AJ001033
Sphaerocarpos texanus Austin	U.S.A., *Goffinet 6476* (DUKE)	AY608110	AY607989	AY608252	—
Stenorrhipis madagascariensis (Steph.) Grolle	Madagascar, *Pocs 9446/AQ* (F)	AY608111	AY607990	AY608253	AY608315
Symphyogyna brogniartii Mont.	Ecuador, *Davis 341* (DUKE)	AY608112	AY607991	AY608254	—
Targionia hypophylla L.	U.S.A., *Buck 15756* (DUKE)	AY608113	AY607992	AY608255	*AJ001001
Telaranea pulcherrima (Steph.) R. M. Schust.	Australia, *Streimann 59554* (NY)	AY608114	AY607993	AY608256	AY608316
Temnoma pulchellum (Hook.) Mitt. ex Bastow	New Zealand, *Braggins 92/87* (F)	AY608115	AY607994	AY608257	AY608317

Table 1. Continued.

Species	Collection	GenBank accession no.			
		*rps*4	*psb*A	26S	*nad*5
Tetralophozia setiformis (Ehrh.) Schljakov	Canada, *Davis 422* (DUKE)	AY608116	AY607995	AY608258	AY608318
Triandrophyllum subtrifidum (Hook. & Taylor) Fulford & Hatcher	Venezuela, *Ricardi, Guaria, Pocs & Pocs 9730/T* (F)	AY608117	AY607996	AY608259	AY608319
Trichocolea tomentosa (Sw.) Gottsche	Ecuador, *Davis 368* (DUKE)	AY608118	AY607997	AY608260	AY608320
Tritomaria quinquedentata (Huds.) H. Buch	U.S.A., *Schofield 106093* (DUKE)	AY608119	AY607998	AY608261	AY608321
OUTGROUP TAXA					
Andreaea rupestris Hedw.		—	—	—	*AJ001227
Andreaea wilsonii Hook. f.	Chile, *Cox 00-668* (DUKE)	*AY330477	*AY312892	AY608194	—
Anthoceros punctatus L.		*AJ250117	—	*AF226036	*AJ000698
Megaceros cf. *fuegiensis* Steph.	Chile, *Cox 00-97* (DUKE)	—	AY607923	—	—
Dendrohypopterygium arbuscula (Brid.) Kruijer	Chile, *Holz & Franzaring CH 00-80* (NY)	AY608059	AY607938	AY608209	AY608276

[1] A. S. Lantratova, V. A. Bakalin, and A. I. Maksimov. 1998. Bryophyta Karelica Exsiccata. Fascicle 1 (1–50).

sonii Hook. f., *Jamesoniella autumnalis* (DC.) Steph. for *Jamesoniella colorata* (Lehm.) Steph., *Lejeunea cavifolia* A. Evans (Ehrh.) Lindb. for *Lejeunea cladogyna* A. Evans, *Plagiochila asplenioides* (L.) Dumort. for *Plagiochila austinii* A. Evans, and *Sphaerocarpos donnelli* Austin for *Sphaerocarpos texanus* Austin. *Megaceros* cf. *fuegiensis* Steph. was sequenced for *psb*A, and combined with GenBank sequences for *Anthoceros punctatus* L. 26S, *rps*4, and *nad*5. Details on voucher specimens and GenBank accession numbers are given in Table 1. This data matrix will be referred to as the "full matrix," and analyses using this matrix referred to as "full analyses," throughout this text.

SAMPLING FOR THE 12-GENE, 26-TAXON ANALYSES: BACKBONE MATRIX

In order to resolve relationships among clades along the backbone of the tree, a subsample of full matrix taxa were chosen to represent each major clade resolved in preliminary analyses of the full matrix, including 23 liverworts and *Dendrohyptopterygium arbuscula* (Brid.) Kruijer. Additional outgroups *Andreaea rothii* F. Weber & D. Mohr and *Hookeria lucens* (Hedw.) Sm. were also added. Sequence data for *18*S nrDNA, *nad*1 and *trn*S mtDNA, and *atp*B, *rbc*L, *psb*T, *trn*G, and *trn*L cpDNA were obtained from GenBank or generated for this study (159 novel sequences). Details on voucher specimens and GenBank accession numbers are given in Table 2. Sequences for these eight loci were combined with sequences from the full matrix to produce a 12-gene matrix for these taxa. This data matrix will be referred to as the "backbone matrix," and analyses using this matrix referred to as "backbone analyses," throughout this text.

SEQUENCING

Total genomic DNA was extracted using a modified version of the method of Doyle and Doyle (1987). All polymerase chain reactions (PCR) were performed using a Perkin Elmer 480 or ThermoHybaid PCR Express. See Table 3 for primers utilized. All PCR amplicons were purified using QIAquick spin-columns according to manufacturer protocols. Sequencing PCR utilized Big Dyes v.2.0 or 3.0 and an ABI PRISM 3700. All sequences are available from GenBank (Tables 1, 2).

Sequences were manually aligned using Se-Al version 2.0a11 (Rambaut, 2002). Regions that could not be unambiguously aligned were excluded from further analysis. The alignments are available from the author upon request.

Table 2. Voucher information and GenBank accession numbers for specimens in the backbone data matrix. Sequences for 26S, *nad*5, *rps*4, and *psb*A for these accessions, except for *Hookeria lucens* and *Andreaea rothii,* were also used in the full data matrix. Non-novel sequences are marked with an asterisk (*). Dashes (—) indicate the no sequence was obtained.

Species	Collection	GenBank accession no.				
		*rps*4	*psb*A	26S	*nad*5	18S
INGROUP TAXA						
Calypogeia muelleriana (Schiffn.) K. Müll.	U.S.A., *Davis 130* (DUKE)	AY608053	AY607932	—	AY608270	AY607849
Ceratolejeunea coarina (Gottsche) Steph.	Brazil, *Zartman 1235.1* (DUKE)	AY608056	AY607935	AY608206	AY608273	AY607850
Dumortiera hirsuta (Sw.) Nees	*Goffinet 6462* (DUKE)	AY608048	AY607927	AY608200	AY608267	AY607852
Gackstroemia weindorferi (Herzog) Grolle	Australia, *Streimann 58531* (NY)	AY608063	AY607943	AY608214	AY608281	AY607853
Haplomitrium blumei (Nees) R. M. Schust.	Guadeloupe, *Goffinet 1904* (CONN)	AY608066	AY607946	AY608216	AY608282	AY607854
Haplomitrium gibbsiae (Steph.) R. M. Schust.	New Zealand, *Engel & von Konrat* 24919 (F)	AY608067	AY607947	—	AY608283	AY607855
Haplomitrium hookeri (Sm.) Nees	U.S.A., *Schofield 95224* (DUKE)	AY608068	*AY312904	*AY330437	AY608284	*U18504
Herbertus sakurai (Warnst.) S. Hatt.	Sargent living culture collection, Berkeley (UC)	AY608066	AY607946	AY608216	AY608282	AY607856
Isotachis lyallii (R. M. Schust.) Mitt.	New Zealand, *Engel 21825* (F)	AY608073	AY607952	AY608221	AY608287	AY607857
Jubula pennsylvanica (Steph.) A. Evans	U.S.A., *Risk 11005* (DUKE)	AY608075	AY607954	AY608223	AY608288	AY607858
Jubulopsis novae-zelandiae (E. A. Hodgs. & S. W. Arnell) R. M. Schust.	New Zealand, *von Konrat 99/Feb. #16* (F)	AY608076	AY607955	AY608224	AY608289	—
Lepidozia reptans (L.) Dumort.	Sargent living culture collection, Berkeley (UC)	AY608083	AY607962	AY608229	AY608293	AY607859
Marsupidium latifolium R. M. Schust.	Costa Rica, *Dauphin 2920* (NY)	AY608088	AY607967	AY608233	AY608298	AY607860
Metzgeria sp. indet.	Ecuador, *Davis 361* (DUKE)	AY608090	AY607969	AY608234	—	AY607861
Odontoschisma denudatum (Nees) Dumort.	U.S.A., *Horn 1809* (DUKE)	AY608096	AY607975	AY608240	AY608303	AY607862
Pellia epiphylla (L.) Corda	U.S.A., *Risk & Gross 12231* (DUKE)	*AY330479	*AY312912	AY608242	AY608305	*X80210
Petalophyllum ralfsii (Wilson) Nees & Gottsche	Sargent living culture collection, Berkeley (UC)	AY608098	AY607977	AY608243	AY608306	AY607863
Pleurozia purpurea Lindb.	U.S.A., *Schofield 102815* (DUKE)	AY608100	AY607979	—	AY608307	AY607864
Porella pinnata L.	U.S.A., *Goffinet 4744* (DUKE)	AY608101	AY607980	AY608245	AY608308	AY607865
Preissia quadrata (Scop.) Nees	U.S.A., *Schofield 105579* (DKE)	AY608102	AY607981	AY608246	AY608309	*X80211
Ptilidium ciliare (L.) Hampe	Canada, *Schofield 103486* (DUKE)	AY608103	AY607982	AY608247	AY608310	AY607866

Table 2. Continued.

Species	GenBank accession no.						
	*trn*S	*nad*1	*psb*T	*rbc*L	*atp*B	*trn*G	*trn*L
INGROUP TAXA							
Calypogeia muelleriana (Schiffn.) K. Müll.	AY608145	AY607870	AY608000	U87065	AY607896	AY608169	AY608121
Ceratolejeunea coarina (Gottsche) Steph.	AY608146	AY607871	AY608001	AY608026	AY607897	AY608170	AY608122
Dumortiera hirsuta (Sw.) Nees	AY608147	AY607873	AY608003	*U87068	AY607899	AY608172	AY608124
Gackstroemia weindorferi (Herzog) Grolle	AY608148	AY607874	AY608004	AY608028	AY607900	AY608173	AY608125
Haplomitrium blumei (Nees) R. M. Schust.	AY608149	AY607875	AY608005	AY608029	AY607901	AY608174	AY608126
Haplomitrium gibbsiae (Steph.) R. M. Schust.	AY608150	AY607876	AY608006	AY608030	AY607902	AY608175	AY608127
Haplomitrium hookeri (Sm.) Nees	AY608151	AY607877	AY608007	*U87072	*AF313555	—	
Herbertus sakurai (Warnst.) S. Hatt.	AY608152	AY607878	AY608008	AY608031	AY607903	AY608176	AY608128
Isotachis lyallii (R. M. Schust.) Mitt.	AY608153	AY607880	AY608010	AY608032	AY607905	AY608178	AY608130
Jubula pennsylvanica (Steph.) A. Evans	AY608154	AY607881	AY608011	*U87074	AY607906	AY608179	AY608131
Jubulopsis novae-zelandiae (E. A. Hodgs. & S. W. Arnell) R. M. Schust.	AY608155	AY607882	AY608012	AY608033	—	AY608180	AY608132
Lepidozia reptans (L.) Dumort.	AY608156	AY607883	AY608013	*U87075	AY607907	AY608181	AY608133
Marsupidium latifolium R. M. Schust.	AY608157	AY607884	AY608014	AY608034	AY607908	AY608182	AY608134
Metzgeria sp. indet.	AY608158	AY607885	AY608015	AY608035	AY607909	AY608183	AY608135
Odontoschisma denudatum (Nees) Dumort.	AY608159	AY607886	AY608016	AY608036	AY607910	AY608184	AY608136
Pellia epiphylla (L.) Corda	AY608160	AY607887	AY608017	*U87085	AY607911	AY608185	AY608137
Petalophyllum ralfsii (Wilson) Nees & Gottsche	AY608161	AY607888	AY608018	*U87086	AY607912	AY608186	AY608138
Pleurozia purpurea Lindb.	AY608162	AY607889	AY608019	AY608037	AY607913	AY608187	AY608139
Porella pinnata L.	AY608163	AY607890	AY608020	*U87088	AY607914	AY608188	AY608140
Preissia quadrata (Scop.) Nees	AY608164	AY607891	AY608021	AY312935	AY607915	—	AY608141
Ptilidium ciliare (L.) Hampe	AY608165	AY607892	AY608022	AY608038	AY607916	AY608189	AY608142
Scapania nemorosa (L.) Dumort.	AY608166	AY607893	AY608023	AY608039	AY607917	AY608190	AY608143
Trichocolea tomentosa (Sw.) Gottsche	AY608167	AY607894	AY608024	AY608040	AY607918	AY608191	AY608144
OUTGROUP TAXA							
Andreaea rothii F. Weber & D. Mohr	—	AY607869	AY607999	AY608025	AY607895	AY608168	AY608120
Dendrohypopterygium arbuscula (Brid.) Kruijer	—	AY607872	AY608002	AY608027	AY607898	AY608171	AY608123
Hookeria lucens (Hedw.) Sm.	—	AY607879	AY608009	*AY312931	AY607904	AY608177	AY608129

Table 3. Primers used for generating sequence data for the backbone and full matrices of the liverwort phylogeny. References are given for published primers; sequences (5′–3′) are given for unpublished primers.

Locus	Primers	Reference or sequence (if unpublished)
18S nrDNA	*NS1, PCRB*	Cox et al. (2000)
	18J, 18Q, 18ERC	Hamby et al. (1988)
26S nrDNA	*0F, 8R, 12R*	Shaw (2000)
*nad*1 mtDNA	*nad1B, nad1C*	Demesure et al. (1995)
*nad*5 mtDNA	*K, Li*	Beckert et al. (1999)
*trn*S mtDNA	*trnSF, trnSR*	Pacak & Szweykowska-Kulinska (2000)
	trnSFi	CTACCYAACCYGGACATGC
	trnSRi	TCACCCATGCTCARG
*rbc*L cpDNA	*NM34, MtmRR*	Cox et al. (2000)
	M1390, M636, M34, M745	Lewis et al. (1997)
*atp*B cpDNA	*atpB672F, atpE384R, atpB493F, atpB1163F, atpB910R*	Pryer et al. (2000)
*psb*A cpDNA	*trnK2F, psbA576R, psbA501F, trnHR*	C. Cox, pers. comm.
*psb*T cpDNA	*psbT, psbH*	Krellwitz et al. (2001)
*trn*G cpDNA	*trnGF, trnGR*	Pacak & Szweykowska-Kulinska (2000)
*trn*L cpDNA	*trnC, trnF*	Taberlet et al. (1991)
*rps*4 cpDNA	*rps5*	Nadot et al. (1994)
	trnas	Cox et al. (2000)

PHYLOGENETIC ANALYSES: PARSIMONY AND LIKELIHOOD METHODS

Aligned sequences were combined and analyzed using equally weighted parsimony (both data matrices) and maximum likelihood (the backbone matrix only) as implemented in PAUP* 4.0b10 (Swofford, 2002). Heuristic parsimony searches were conducted using 300 random taxon addition replicates with the MulTrees option in effect and TBR branch-swapping. Gaps were scored as missing data. Parsimony bootstrap support values were calculated using a heuristic search with 300 (backbone matrix) or 100 (full matrix) replicates and 10 random taxon addition sequences per replicate (Felsenstein, 1985).

For parsimony analyses of the full matrix, the relationships among the major clades were constrained to the topology obtained from analyses on the backbone matrix. Relationships within clades were not specified: the PAUP* block constraint tree specified was (((((*Ceratolejeunea coarina, Gackstroemia weindorferi*), (*Calypogeia muelleriana, Scapania nemorosa*)), *Metzgeria*), *Petalophyllum ralfsii*), (*Haplomitrium blumei, Haplomitrium gibbsiae, Haplomitrium hookeri*)).

For maximum likelihood analyses of the backbone matrix, the optimal nucleotide substitution model was chosen using the hierarchical likelihood ratio test as implemented in Modeltest 3.06 (Posada & Crandall, 1998). Parameters utilized for the likelihood analysis are shown in Table 4 (single partition). Maximum likelihood searches under the optimal model were conducted using a neighbor-joining starting tree with MulTrees in effect and TBR swapping. Likelihood bootstrap support values were calculated using a fast-heuristic search with 300 random taxon addition replicates.

Although it was not utilized for maximum likelihood reconstruction analyses due to the computational time required, the optimal nucleotide substitution model for the full matrix was also chosen using Modeltest. This substitution model was specified for a homogeneous Bayesian analysis (Table 5, single partition).

PHYLOGENETIC ANALYSES: HOMOGENEOUS AND HETEROGENEOUS BAYESIAN METHODS

It is desirable to include various genetic compartments in analyses, in order to build a robust phylogenetic hypothesis; however, heterogeneous patterns of nucleotide substitution among compartments can create conflict among hypotheses. Such compartmental heterogeneity may be expected when comparing coding and non-coding sequences, intron and exon sequences, first, second, and third positions, or sequences from multiple genomes (i.e., nuclear, chloroplast, mitochondrial). Accommodating such heterogeneous data in a single analysis is problematic. Maximum likelihood analyses generally apply a single substitution model across a data set, even when that data set consists of partitions that differ in their patterns of substitution. The ap-

plication of multiple, heterogenous sets of parameters for partitioned data is theoretically possible in maximum likelihood analyses, but such analyses are so computationally intensive that they are impossible in practice. However, model-based phylogenetic methods using Bayesian inference dramatically decrease the computational effort required (Lewis, 2001).

Homogeneous Bayesian analyses are analogous to single model maximum likelihood; a single model is applied across an entire data set. Homogeneous analyses are useful alternatives to maximum likelihood analyses when there are many taxa included, because they require much shorter computation times. Heterogeneous Bayesian analyses apply multiple, heterogenous sets of parameters to partitioned data within a data set. Both types of Bayesian methods were applied in the current study.

The substitution models chosen by Modeltest for the 4- and 12-gene data sets were specified for homogeneous Bayesian analyses (Tables 4, 5). Six replicates of homogeneous model analyses were conducted on each data matrix using MrBayes 3.0b (Huelsenbeck & Ronquist, 2003). For each replicate, four simultaneous Markov chain Monte Carlo chains were run for 200,000 generations (three chains heated) and parameters were sampled every 10 generations. Plots of the likelihoods from each sample were made to determine the number of generations until stationarity was achieved, in order to identify the posterior probability tree set; trees sampled before the onset of stationarity (i.e., burn-in) were removed.

Each data matrix was partitioned for purposes of heterogeneous model Bayesian analyses. Partitions were specified for an entire locus (e.g., *rps*4), or a specific codon position within a coding locus (e.g., *rps*4 1st). While each heterogeneous analysis actually optimized a single model, that model was composed of submodels for data partitions; for simplicity these will be referred to as 4-model for a model composed of 4 submodels, or 12-model for a model composed of 12 submodels, and so on. For the backbone matrix, 12-model (partitions defined for each locus) and 21-model (partitions defined for *18*S, *26*S, *nad*1, *nad*5, *trn*S, *atp*B 1st, *atp*B 2nd, *atp*B 3rd, *rps*4 1st, *rps*4 2nd, *rps*4 3rd, *psb*A 1st, *psb*A 2nd, *psb*A 3rd, *psb*A non-coding, *rbc*L 1st, *rbc*L 2nd, *rbc*L 3rd, *psb*T, *trn*L, and *trn*G) analyses were conducted. For the full matrix, 4-model (partitions defined for each locus) and 9-model (partitions defined for *26*S, *nad*5, *rps*4 1st, *rps*4 2nd, *rps*4 3rd, *psb*A 1st, *psb*A 2nd, *psb*A 3rd, and *psbA* non-coding) analyses were conducted. The maximum likelihood substitution model for each partition was chosen using the hierarchical likelihood ratio test as implemented in the program Modeltest 3.06. See Table 4 for the models chosen for each partition in the backbone matrix; Table 5 lists models chosen for each partition in the full matrix. Six replicate analyses for each of the heterogeneous models were conducted using MrBayes 3.0b. For each replicate, four simultaneous Markov chain Monte Carlo searches were run for 200,000 generations (three chains heated) with parameters and trees sampled every 10 generations. Plots of the likelihoods from each sample were made to determine the number of generations until stationarity was achieved in order to identify the posterior probability tree set; burn-in trees were removed.

Analyses of the full matrix were subject to instability along the backbone; therefore, in parsimony analyses on the full matrix, the topology was constrained to the backbone tree (see Phylogenetic analyses: parsimony and likelihood methods, above). During data exploration, it was determined that the ambiguous placement of *Haplomitrium* was a major contributor to this instability. Therefore, to avoid the associated instability, *Haplomitrium* was excluded from Bayesian analyses of the full data matrix. Backbone analyses demonstrated that *Haplomitrium* is not a member of the leafy liverwort clade (see results); furthermore, phylogenetic relationships within the leafy liverworts were not sensitive to *Haplomitrium*'s placement in the backbone analysis (data not shown).

PHYLOGENETIC ANALYSES: TESTING SPECIFIC HYPOTHESES

The leafy liverwort *Ptilidium ciliare* (L.) Hampe was resolved in a different clade in the full analyses than in the backbone analyses. To further examine the phylogenetic placement of *Ptilidium*, comparisons were made between sets of trees using the Templeton and the Shimodaira-Hasegawa (S-H) tests as implemented in PAUP* (Swofford, 2002). Comparisons were made among 10 randomly chosen trees from the set of most parsimonious trees when (1) the full data matrix was constrained to the backbone tree but *Ptilidium* was unconstrained, and (2) the full data matrix and *Ptilidium* were constrained to the backbone tree.

RESULTS

BACKBONE ANALYSES

Parsimony analysis yielded one tree (2208 parsimony informative characters, length = 9663, con-

Table 4. Model selection results for each partition in the backbone data matrix. The model was chosen using the hierarchical likelihood ratio test as implemented in the program Modeltest (Posada & Crandall, 1998). (GTR: General Time Reversible, Rodriguez et al., 1990; HKY: Hasegawa-Kishino-Yano, Hasegawa et al., 1985; SYM: model of Zharkikh, 1994; K80: Kimura, 1980). 1st = First codon positions, 2nd = second codon positions, and 3rd = third codon positions. Base = base frequencies (A, C, G); Rmat = rate matrix (AC, AG, AT, CG, CT); TRatio = transversion ratio; Shape = value of ∀ in gamma distribution; Pinvar and proportion of invariant characters.

Partition	−ln Likelihood	Model name	Parameter values
single	56796.0742	TrN+I+G	Base = (0.2842.0.1993 0.2227) Nst = 6 Rmat = (1.0000 5.0849 1.0000 1.0000 7.5834) Rates = gamma Shape = 0.5714 Pinvar = 0.4108
18S	4315.8667	TrN+I+G	Base = (0.2250 0.2087 0.2776) Nst = 6 Rmat = (1.0000 2.3390 1.0000 1.0000 6.2672) Rates = gamma Shape = 0.5597 Pinvar = 0.5879
26S	3804.8547	TrN+I+G	Base = (0.2417 0.2384 0.3285) Nst = 6 Rmat = (1.0000 3.2924 1.0000 1.0000 8.5566) Rates = gamma Shape = 0.6006 Pinvar = 0.5019
*Nad*1	778.6974	GTR+G	Base = (0.2074 0.2021 0.2004) Nst = 6 Rmat = (6.8125 4.8496 0.0000 0.7550 19.8542) Rates = gamma Shape = 0.4031 Pinvar = 0
*Nad*5	3515.5017	TIM+G	Base = (0.2698 0.2263 0.1912) Nst = 6 Rmat = (1.0000 2.8167 0.3185 0.3185 4.6998) Rates = gamma Shape = 0.4691 Pinvar = 0
*trn*S	3690.6936	K81uf+G	Base = (0.2636 0.2304 0.2781) Nst = 6 Rmat = (1.0000 3.8795 0.5168 0.5168 3.8795) Rates = gamma Shape = 0.5638 Pinvar = 0
*atp*B	8391.4229	TrNef+I+G	Base = equal Nst = 6 Rmat = (1.0000 4.6531 1.0000 1.0000 5.7685) Rates = gamma Shape = 0.8773 Pinvar = 0.4475
*atp*B 1st	1296.3623	GTR+G	Base = (0.2495 0.1735 0.3951) Nst = 6 Rmat = (9.1714 2.4064 0.5440 1.2034 22.7826) Rates = gamma Shape = 0.1969 Pinvar = 0
*atp*B 2nd	820.1927	HKY+G	Base = (0.2782 0.2683 0.1532) Nst = 2 TRatio = 3.9683 Rates = gamma Shape = 0.1184 Pinvar = 0
*atp*B 3rd	5486.2007	GTR+I+G	Base = (0.4042 0.1030 0.0981) Nst = 6 Rmat = (1.1833 6.9430 0.0153 1.9316 6.3569) Rates = gamma Shape = 2.8606 Pinvar = 0.0548
*Rbc*L	9697.04	GTR+I+G	Base = (0.2697 0.1708 0.2282) Nst = 6 Rmat = (3.0814 6.9094 1.4651 2.9887 15.8496) Rates = gamma Shape = 1.3403 Pinvar = 0.4929
*Rbc*L 1st	1788.134	TrN+I+G	Base = (0.2411 0.2450 0.3496) Nst = 6 Rmat = (1.0000 0.4994 1.0000 1.0000 6.3779) Rates = gamma Shape = 0.5333 Pinvar = 0.5016
*Rbc*L 2nd	1150.5522	JC+G	Base = equal Nst = 1 Rates = gamma Shape = 0.1584 Pinvar = 0
*Rbc*L 3rd	6030.9312	TVM+I+G	Base = (0.3306 0.1129 0.0870) Nst = 6 Rmat = (1.2862 8.6307 0.2354 1.3807 8.6307) Rates = gamma Shape = 3.2691 Pinvar = 0.0523
*Rps*4	6628.4546	HKY+I+G	Base = (0.3546 0.1655 0.1915) Nst = 2 TRatio = 3.5219 Rates = gamma Shape = 1.1328 Pinvar = 0.2221
*Rps*4 1st	1763.1246	K81uf+G	Base = (0.3716 0.2074 0.2111) Nst = 6 Rmat = (1.0000 3.7511 0.3076 0.3076 3.7511) Rates = gamma Shape = 0.5448 Pinvar = 0
*rps*4 2nd	1402.6674	K80+G	Base = equal Nst = 2 TRatio = 3.7001 Rates = gamma Shape = 0.4029 Pinvar = 0
*Rps*4 3rd	3191.9187	TVM+I+G	Base = (0.3816 0.0685 0.1064) Nst = 6 Rmat = (1.2671 8.8411 0.1959 6.9752 8.8411) Rates = gamma Shape = 8.3297 Pinvar = 0.0221
*psb*A	6623.271	GTR+I+G	Base = (0.2417 0.1997 0.2037) Nst = 6 Rmat = (1.2481 9.3073 2.7206 0.3940, 19.9978) Rates = gamma Shape = 1.2330 Pinvar = 0.5816

Table 4. Continued.

Partition	−ln Likelihood	Model name	Parameter values
*psb*A 1st	1850.6613	TrN+I+G	Base = (0.2606 0.1638 0.2443) Nst = 6 Rmat = (1.0000 5.6886 1.0000 1.0000 18.4066) Rates = gamma Shape = 1.9519 Pinvar = 0.6777
*psb*A 2nd	711.3275	TrNef+I+G	Base = equal Nst = 6 Rmat = (1.0000 5.1992 1.0000 1.0000 14.6135) Rates = gamma Shape = 0.5405 Pinvar = 0.7549
*psb*A 3rd	2944.8516	GTR+I+G	Base = (0.2473 0.2196 0.1101) Nst = 6 Rmat = (0.3435 8.4901 1.3513 0.5089 9.4049) Rates = gamma Shape = 2.6383 Pinvar = 0.3500
*psb*A non-coding	770.2067	HKY+G	Base = (0.3817 0.1358 0.1153) Nst = 2 TRatio = 3.2900 Rates = gamma Shape = 0.4010 Pinvar = 0
*psb*T	3077.3091	HKY+I+G	Base = (0.3448 0.1635 0.1928) Nst = 2 TRatio = 4.1165 Rates = gamma Shape = 0.9718 Pinvar = 0.3589
*trn*G	2012.7797	HKY+G	Base = (0.3320 0.2046 0.1064) Nst = 2 TRatio = 3.1273 Rates = gamma Shape = 0.8758 Pinvar = 0
*trn*L	1791.33	HKY+I+G	Base = (0.3159 0.1628 0.2215, Nst = 2 TRatio = 3.1825 Rates = gamma Shape = 0.8112 Pinvar = 0.3697

sistency index, CI = 0.493); maximum likelihood analyses yielded the same tree (−ln likelihood = 57,972.45; Fig. 1).

The topologies for homogeneous and heterogeneous 12-model analyses match that shown in Figure 1. The only significant difference between the results of these analyses and the 21-model heterogeneous analyses concerned the placement of *Haplomitrium*; in the latter, the *Haplomitrium* clade is part of the first dichotomy in liverworts and is sister to the rest of the liverworts.

The topology was robust to differing analytical methods (maximum parsimony, maximum likelihood, homogeneous and heterogeneous Bayesian). Most nodes were consistently highly supported (Table 6, Fig. 1). Liverworts are resolved as monophyletic with maximum support; however, the first branching lineage within the liverworts is ambiguous. The marchantioids are sister to the clade including *Haplomitrium* plus the jungermannioids in the parsimony, likelihood, and homogeneous analyses with significant support (bootstrap value (BS) = 97, BS = 80, and posterior probability = 97%, respectively; Fig. 1). This topology was also obtained in the 12-model heterogeneous Bayesian analysis, although with nonsignificant support (posterior probability = 89%; Fig. 1). In the 21-model heterogeneous Bayesian analysis, however, *Haplomitrium* is sister to the clade composed of the rest of the liverworts, although without significant support (posterior probability = 63%; tree not shown).

The simple thalloid liverworts are paraphyletic and composed of two clades (Fig. 1). The first clade (simple thalloid I) is represented in this analysis by *Pellia epiphylla* (L.) Corda and *Petalophyllum ralfsii* (Wilson) Nees & Gottsche, while the second clade (simple thalloid II) is represented by *Metzgeria* indet. and *Pleurozia purpurea* Lindb. Simple thalloid clade II is sister to the leafy liverwort clade. The leafy liverworts are composed of two monophyletic groups, one containing most of the complicate-bilobed taxa (Leafy I) and one composed of the remaining taxa (Leafy II). While *Ptilidium* is resolved as a member of the Leafy I clade, there is no support for this arrangement in any of the analyses. The unique leafy taxon *Pleurozia* is not in either clade of the Jungermanniidae (the core leafy liverworts); rather, it is resolved with *Metzgeria* (Simple thalloid II) with strong support (parsimony BS = 93, likelihood BS = 68, posterior probability = 100%; Fig. 1).

FULL ANALYSES

Parsimony analysis (constrained to the backbone tree, *Ptilidium* unconstrained) yielded 39 trees (1216 parsimony informative characters, length = 7750, CI = 0.334; tree not shown). All replicates within homogeneous and heterogenous Bayesian analyses converged on a common topology. Homogeneous and heterogeneous Bayesian consensus trees and strict parsimony consensus trees were completely congruent at the level of major clades. The 95% majority rule topology from the homogeneous analysis is shown in Figure 2.

Bayesian analyses resolved the same major clades as the backbone analyses: Leafy I, Leafy II, Simple thalloid I, Simple thalloid II, and Complex

Table 5. Model selection results for each partition in the full data matrix. The model was chosen using the hierarchical likelihood ratio test as implemented in the program Modeltest (Posada & Crandall, 1998). (GTR: General Time Reversible, Rodriguez et al., 1990; HKY: Hasegawa-Kishino-Yano, Hasegawa et al., 1985; SYM: model of Zharkikh, 1994; K80: Kimura, 1980). 1st = First codon positions, 2nd = second codon positions, and 3rd = third codon positions. Base = base frequencies (A, C, G); Rmat = rate matrix (AC, AG, AT, CG, CT); TRatio = transversion ratio; Shape = value of ∀ in gamma distribution; Pinvar and proportion of invariant characters.

Partition	−ln Likelihood	Model name	Parameter values
single	42711.0742	TrN+I+G	Base = (0.2733 0.2224 0.2113) Nst = 6 Rmat = (1.0000 6.2829 1.0000 1.0000 6.6446) Rates = gamma Shape = 0.6308 Pinvar = 0.3486
26S	6988.2173	TrN+I+G	Base = (0.2882 0.2446 0.3435) Nst = 6 Rmat = (1.0000 2.9204 1.0000 1.0000 7.0599) Rates = gamma Shape = 0.4015 Pinvar = 0.3051
*nad*5	6536.7324	GTR+I+G	Base = (0.2676 0.2056 0.2004) Nst = 6 Rmat = (2.2412 4.2149 0.3376 1.1695 5.3477) Rates = gamma Shape = 0.9866 Pinvar = 0.2272
*Rps*4	13452.0596	GTR+I+G	Base = (0.3856 0.1398 0.1499) Nst = 6 Rmat = (1.1530 6.6535 0.2456 1.6853 5.4891) Rates = gamma Shape = 1.0845 Pinvar = 0.2022
*Rps*4 1st	5337.7256	TVM+I+G	Base = (0.4000 0.1105 0.1401) Nst = 6 Rmat = (1.2927 6.3153 0.2079 1.6643 6.3153) Rates = gamma Shape = 0.9683 Pinvar = 0.1506
*Rps*4 2nd	3850.4919	HKY+I+G	Base = (0.3321 0.2326 0.2039) Nst = 2 TRatio = 3.2155 Rates = gamma Shape = 1.2326 Pinvar = 0.1933
*Rps*4 3rd	4075.7869	GTR+I+G	Base = (0.3636 0.1720 0.1519) Nst = 6 Rmat = (0.9100 7.8126 0.2583 1.3855 3.6708) Rates = gamma Shape = 1.1787 Pinvar = 0.2329
*psb*A	12398.9072	GTR+I+G	Base = (0.2422 0.2009 0.1834) Nst = 6 Rmat = (1.2967 11.6273 2.1118 0.7344 15.5753) Rates = gamma Shape = 0.9413 Pinvar = 0.5421
*psb*A 1st	4324.4116	TrN+I+G	Base = (0.2748 0.2220 0.1378) Nst = 6 Rmat = (1.0000 9.6129 1.0000 1.0000 7.6035) Rates = gamma Shape = 0.4371 Pinvar = 0.4614
*psb*A 2nd	6383.4585	GTR+I+G	Base = (0.2472 0.1837 0.1443) Nst = 6 Rmat = (1.7505 15.0252 2.2446 0.8072 14.2947) Rates = gamma Shape = 1.3301 Pinvar = 0.4341
*psb*A 3rd	4322.7842	TrN+I+G	Base = (0.2936 0.1727 0.1926) Nst = 6 Rmat = (1.0000 5.9951 1.0000 1.0000 10.8224) Rates = gamma Shape = 0.9139 Pinvar = 0.5417
*psb*A non-coding	1295.6610	HKY+G	Base = (0.3817 0.1358 0.1153) Nst = 2 TRatio = 3.2900 Rates = gamma Shape = 0.4010 Pinvar = 0

thalloid (Fig. 2). Three large monophyletic groups exist within the Leafy II clade (A, B, and C; Fig. 2). At the base of Leafy II clade is a grade of three lineages (*Temnoma pulchellum* (Hook.) Mitt. ex Bastow, *Schistochila lehmanniana* (Lehm. & Lindenb.) Carrington & Pearson, and *Ptilidium ciliare* + *Neotrichocolea* bisseti (Mitt.) S. Hatt.). This placement for *Ptilidium* is in contrast to the backbone analysis, where it was resolved with the Leafy I clade. Parsimony analyses on the full data matrix where *Ptilidium* was constrained with Leafy I yielded 104 trees (length = 7757); comparisons between these two sets of trees using Templeton and S-H tests were non-significant. These results indicate that the alternative placement of *Ptilidium* within the Leafy I clade cannot be rejected.

Only one notable difference occurred between the strict parsimony consensus tree and the Bayesian trees: while the Bayesian analysis places *Goebeliella cornigera* (Mitt.) Steph., *Lepidolaena* sp. indet., *Jubulopsis novae-zelandiae* (E. A. Hodgs. & S. W. Arnell) R. M. Schust., and *Gackstroemia weindorferi* (Herzog) Grolle as a monophyletic group (Fig. 2), the parsimony consensus tree resolves *Ascidiota blepharophylla* Steere & R. M. Schust. + *Porella pinnata* L. nested within this clade, and

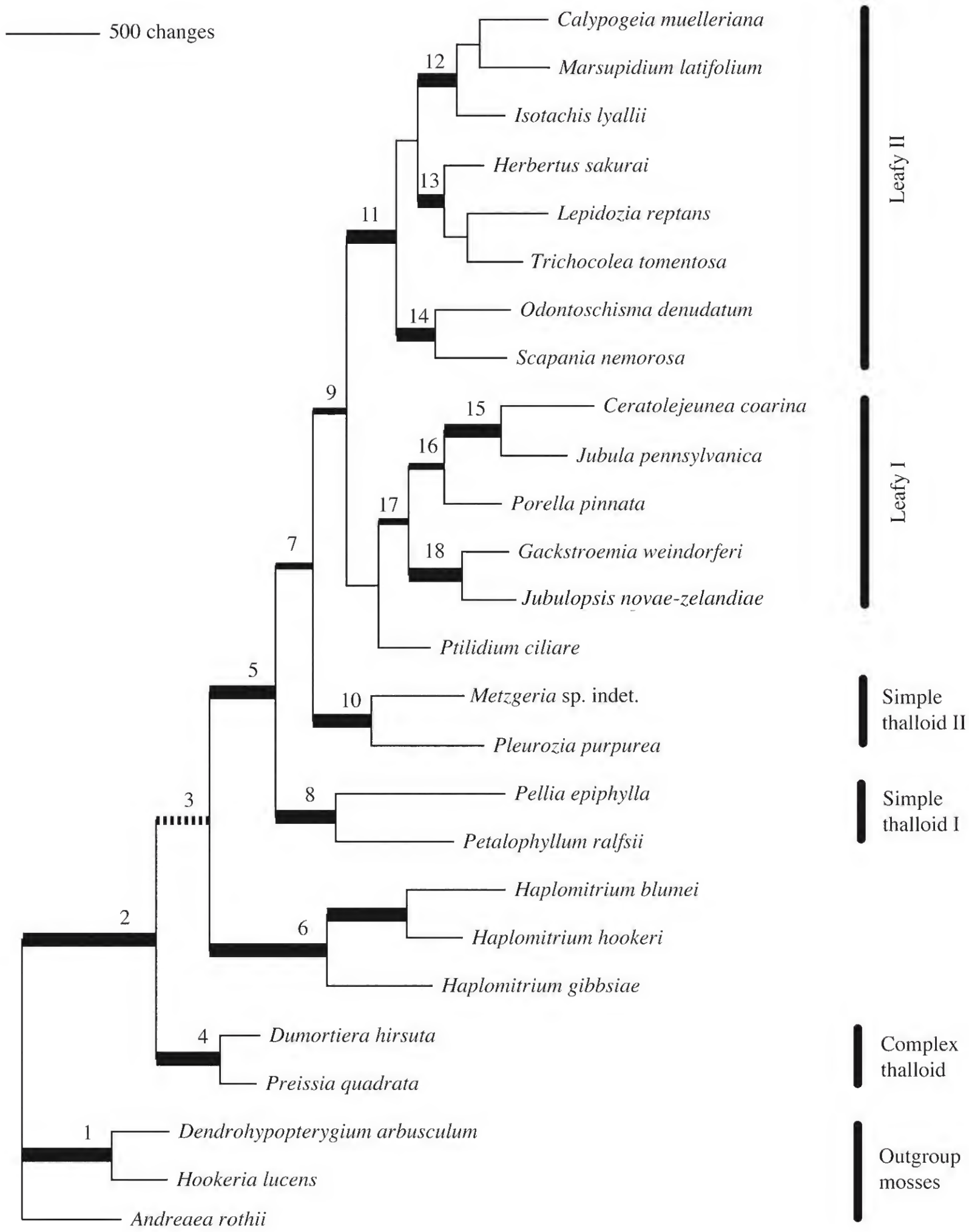

Figure 1. Maximum likelihood tree obtained from the 12-gene backbone data matrix. Double-bold branches indicate support for the clade in all analyses (parsimony and likelihood bootstrap ≥ 60, Bayesian posterior probabilities ≥ 95). Single-bold branches indicate significant support for the clade in Bayesian analyses only (posterior probability ≥ 95). Broken single-bold branch indicates uncertainty in the placement of *Haplomitrium,* which is supported with significant support in the position shown in parsimony, likelihood, and homogeneous Bayesian analyses, but not in heterogeneous Bayesian analyses. Numbers refer to nodes in Table 6; see this table for support values.

Table 6. Support values for nodes labeled on Figure 1 based on maximum parsimony (MP) and maximum likelihood (ML) bootstrap values (≥ 50); and homogeneous (1M), 12-model (12M), and 21-model (21M) heterogeneous Bayesian posterior probabilities.

Node	MP	ML	1M	12M	21M
1	100	100	100	100	100
2	100	100	100	100	100
3	97	80	97	89	63
4	100	100	100	100	100
5	61	61	100	100	100
6	100	100	100	100	100
7	—	—	100	100	100
8	100	98	100	100	100
9	—	—	100	100	100
10	93	68	100	99	99
11	94	90	100	100	100
12	94	92	100	100	100
13	90	90	100	100	100
14	100	100	100	100	100
15	100	100	100	100	100
16	—	—	99	100	100
17	—	—	100	100	100
18	94	92	100	100	100

sister to *Goebeliella* (tree not shown). This topology, however, has no bootstrap support (< 50).

Several nodes were resolved consistently in all analyses (strict parsimony consensus, homogeneous and heterogeneous Bayesian), yet received less than significant support and thus are not shown in the 95% majority rule tree (Fig. 2): *Trichocolea* indet. as the first-branching lineage in Leafy II subclade A, with *Plagiochila austinii* A. Evans + *Chiloscyphus appalachianus* (R. M. Schust.) J. J. Engel & R. M. Schust. sister to the rest of the subclade; in Leafy II subclade C, *Anthelia julacea* (L.) Dumort. as sister to *Calypogeia muelleriana* (Schiffn.) K. Müll. + *Lophocolea* indet., and *Harpanthus scutatus* (F. Weber & D. Mohr) Spruce as sister to the *Blepharostoma trichophyllum* (L.) Dumort. through *Nardia scalaris* Gray clade; in Leafy I clade, *Cyclolejeunea convexistipa* (Lehm. & Lindeng.) A. Evans sister to *Lejeunea cladogyna* A. Evans, and *Frullania* indet. sister to the *Bryopteris filicina* (Sw.) Nees through *Odontolejeunea lunulata* (Nees) Schiffn. clade. The tree shown in Figure 2 resolves *Adelanthus lindenbergianus* (Lehm.) Mitt. as sister to the rest of Leafy II B; this arrangement was supported in all analyses except the 21-model heterogeneous analysis, where it dropped to an unresolved position among clades A, B, and C.

The strict consensus parsimony tree, which included the *Haplomitrium* sequences, resolved the *Haplomitrium* clade as sister to all other liverworts (tree not shown), however, the parsimony bootstrap analysis does not support this arrangement. Some bootstrap support (68; tree not shown) was obtained for *Haplomitrium* as sister to the simple thalloid plus the leafy clades.

Discussion

comparison between classifications and phylogeny

Summaries of the two most recent classifications of leafy liverworts (Schuster, 1984; Crandall-Stotler & Stotler, 2000) are shown in Tables 7 and 8. These two classifications differ in four principal ways: (1) Crandall-Stotler and Stotler segregated five orders, whereas Schuster circumscribed only one; (2) the order Lepicoleales begins the Crandall-Stotler and Stotler classification, while in the Schuster classification, corresponding suborders (Herbertineae, Lepicoleineae, Personiellineae, Ptilidiineae, Lepidolaenineae) are dispersed at the beginning, in the middle, and toward the end; (3) Crandall-Stotler and Stotler divided Schuster's suborder Herbertineae, with part (the Trichocoleaceae) being transferred to the suborder Lepidolaenineae in the Lepicoleales; and (4) Crandall-Stotler and Stotler segregated the Jubulineae out of Schuster's Porellineae.

Of the suborders circumscribed by Schuster (1984) that can be evaluated based on the molecular phylogeny presented here, only the Balantiopsidineae are monophyletic (Fig. 3). Of the orders circumscribed by Crandall-Stotler and Stotler (2000), none (except the monogeneric Radulales) forms a clade (Fig. 4), and of the suborders that can be evaluated based on the phylogeny, the Porellineae and Balantiopsidineae alone are monophyletic. Surprisingly, because *Pleurozia* falls outside of the core leafy clade in the present analyses, Schuster's order Jungermanniales as well as Crandall-Stotler and Stotler's subclass Jungermanniidae appear non-monophyletic.

The five-order system of Crandall-Stotler and Stotler (2000) does not reflect the phylogeny presented here. In particular, the Lepicoleales is extensively polyphyletic, and the Radulales is nested within the Porellales. Schuster (1984) classified the Lepidolaenineae next to the Porellineae, based on endosporic germination and dominantly epiphytic habit; this grouping appears to better reflect phylogenetic relationships revealed by the present study. The recognition of an order Lepicoleales obscures this relationship. The separation of the Jubulineae from the Porellineae (Crandall-Stotler &

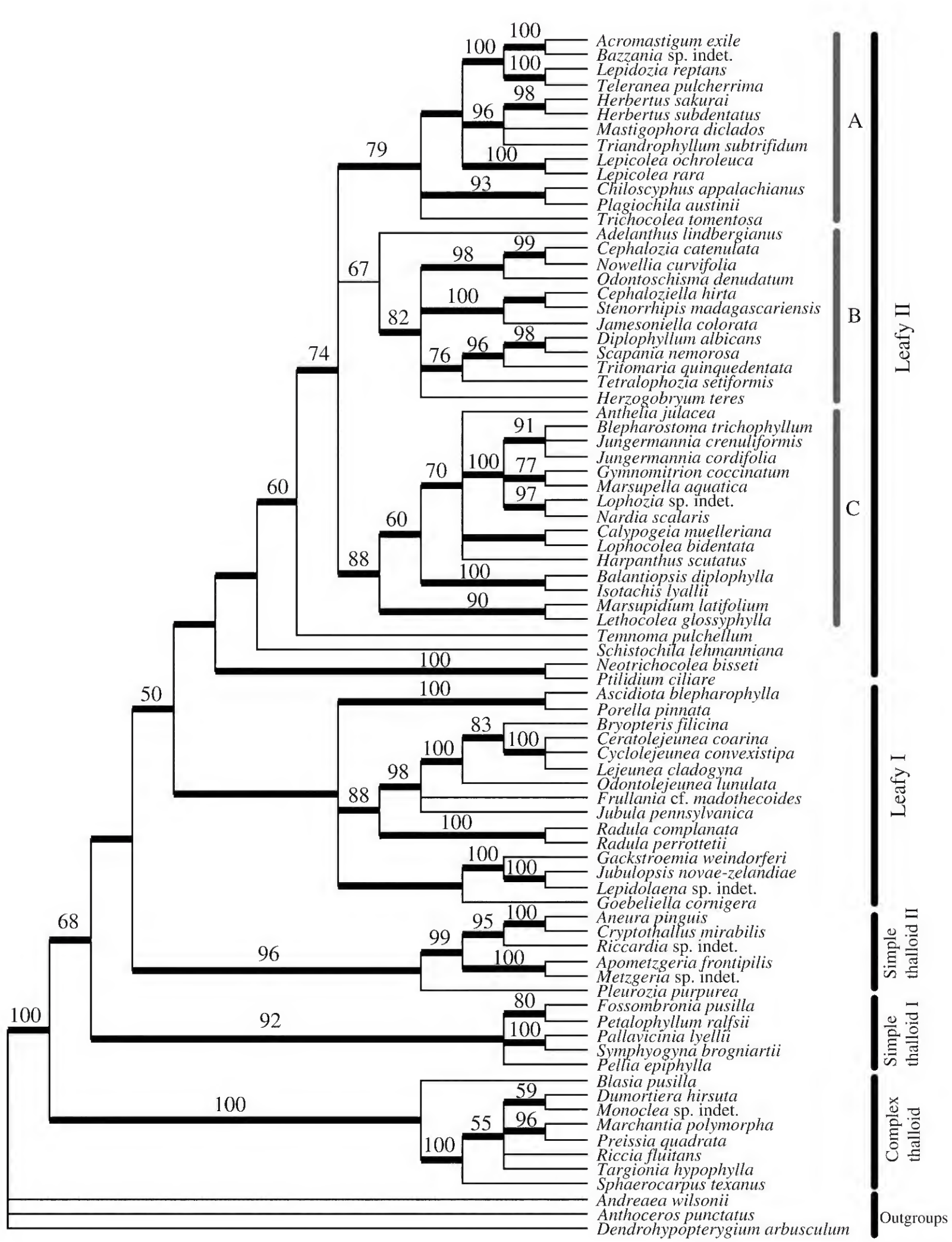

Figure 2. Homogeneous Bayesian 95% majority rule tree obtained from the 4-gene full data matrix (78,006 total trees). Bold branches indicate significant support for the clade in all Bayesian analyses (homogeneous and heterogeneous posterior probabilities ≥ 95). Parsimony bootstrap values ≥ 50 are shown on the tree. Vertical bars and corresponding labels refer to clades discussed in the text.

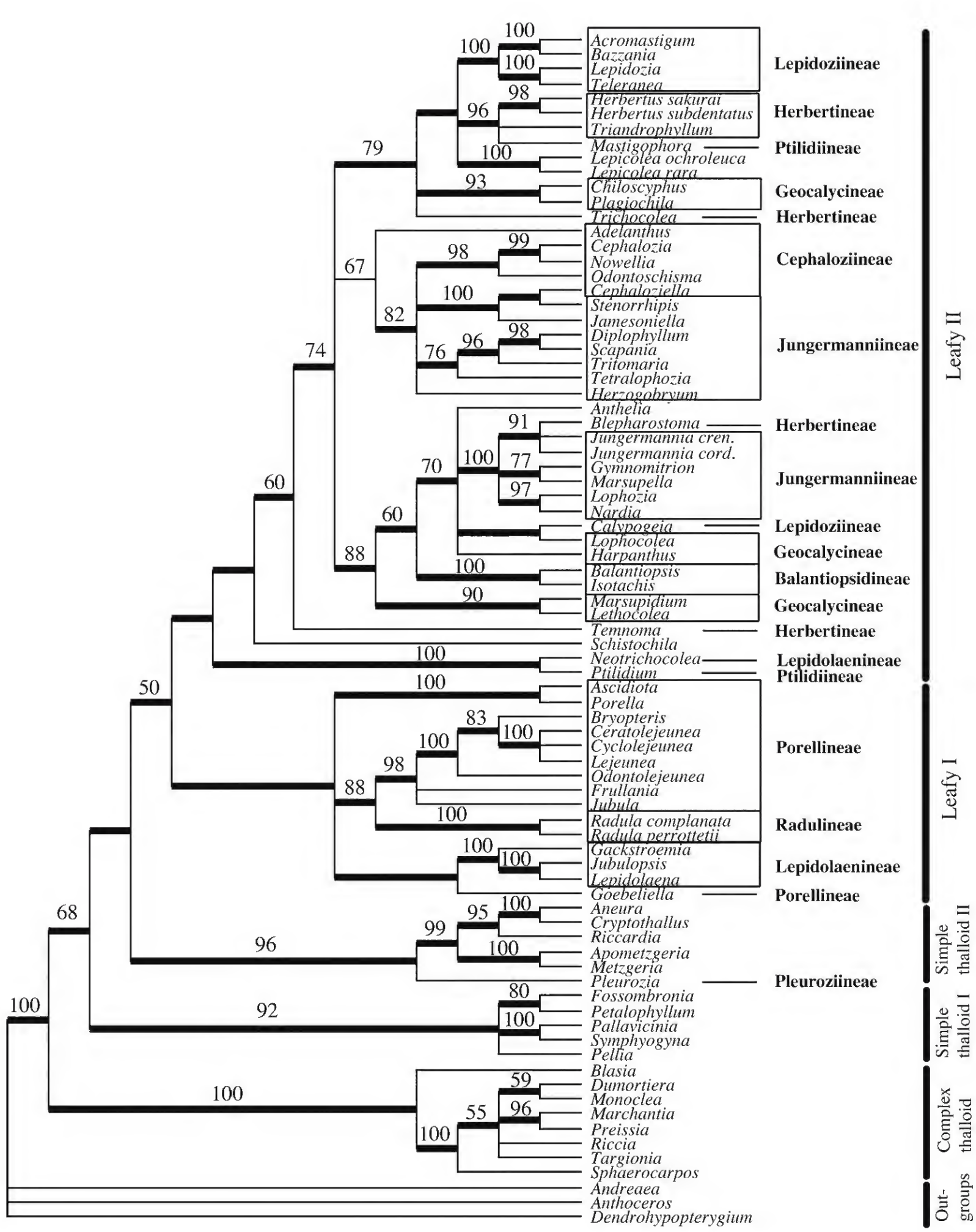

Figure 3. Phylogenetic comparison with subordinal groupings according to Schuster (1984). Tree shown is the homogeneous Bayesian 95% majority rule tree obtained from the 4-gene full data matrix (Fig. 2). Vertical bars and corresponding labels refer to clades discussed in text (Fig. 2).

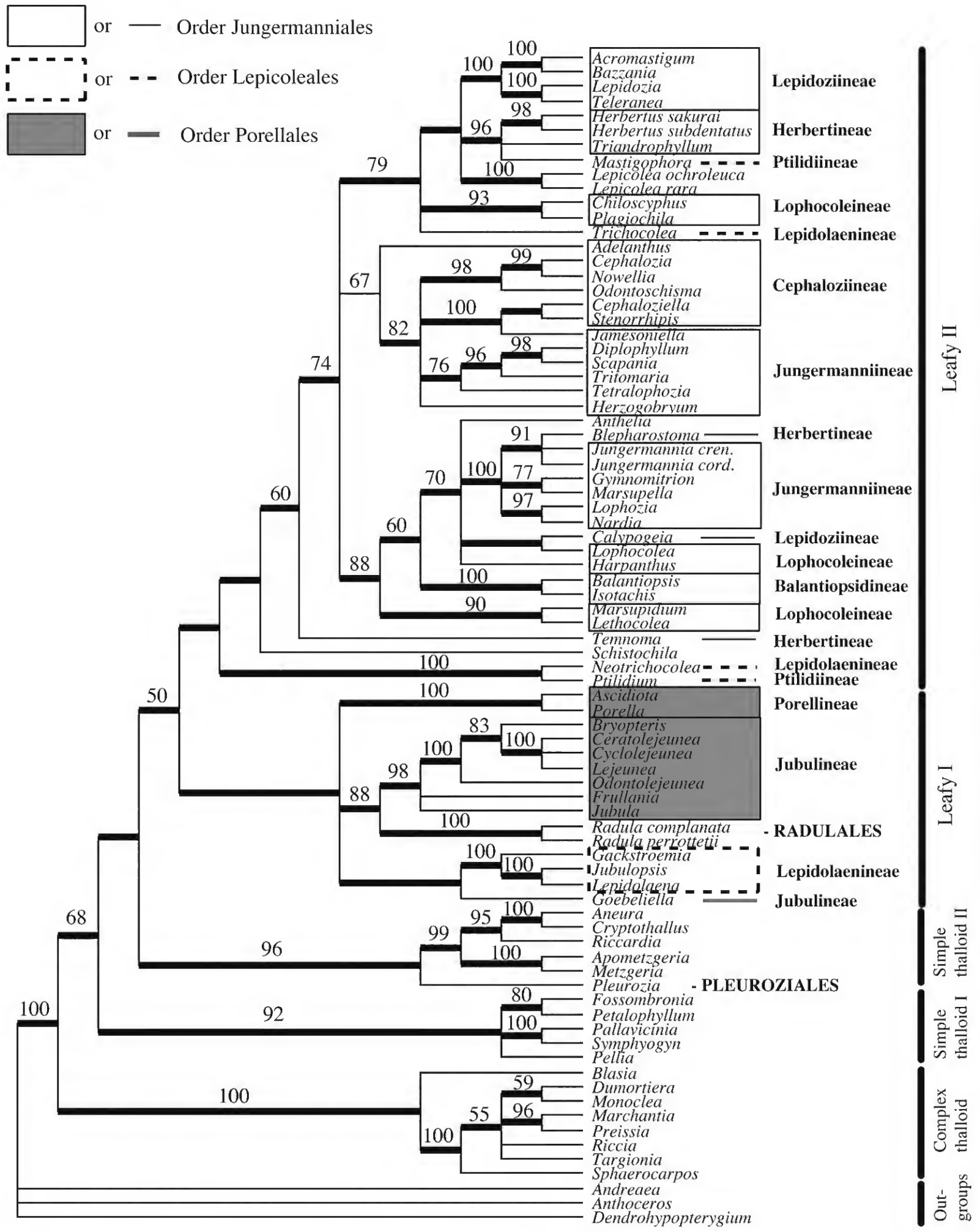

Figure 4. Phylogenetic comparison with ordinal and subordinal groupings according to Crandall-Stotler and Stotler (2000). Order Lepicoleales is represented by dashed lines, Jungermanniales by solid lines, and Porellales by a grey line or shading. Radulales and Pleuroziales are labeled in all caps. Tree shown is the homogeneous Bayesian 95% majority rule tree obtained from the 4-gene full data matrix (Fig. 2). Vertical bars and corresponding labels refer to clades discussed in text (Fig. 2).

Stotler, 2000) more closely reflects the phylogeny than a single Porellineae (Schuster, 1984). Among the other polyphyletic taxa, such as the Herbertineae, Jungermanniineae, and the Geocalycineae/Lophocoleineae, the phylogeny presented here approaches agreement with neither classification.

Of the familial classifications that can be evaluated using the molecular phylogenies in this study, the Herbertaceae, Lepidoziaceae, Balantiopsidaceae, Cephaloziaceae, Acrobolbaceae, and Porellaceae (with the same circumscriptions in both classifications) appear monophyletic. The Lejeuneaceae, with the Bryopteridaceae segregated (sensu Crandall-Stotler & Stotler, 2000) are not monophyletic, but form a clade including *Bryopteris* (sensu Schuster, 1984). The Scapaniaceae, Blepharidophyllaceae, and Delavayellaceae are segregated into separate families by Schuster (1984), but included into a single Scapaniaceae by Crandall-Stotler and Stotler (2000); in the former case, Scapaniaceae is monophyletic, and in the latter, not enough information is available to evaluate the monophyly of the Scapaniaceae. Schuster (2000) recognized a single family, Trichocoleaceae, while Crandall-Stotler and Stotler segregated the Pseudolepicoleaceae from it; in both cases, the Trichocoleaceae are non-monophyletic, and in the latter, the Pseudolepicoleaceae is also non-monophyletic. There is not enough information to determine whether the Jubulaceae form a clade; however, a monophyletic Jubulaceae cannot be rejected. Clearly paraphyletic families in both classifications include the Jungermanniaceae, Gymnomitriaceae, Geocalycaceae, Cephaloziellaceae, and Lepidolaenaceae.

GENERAL SUMMARY OF CLADE CHARACTERISTICS

Some generalizations and tendencies can be noted with regard to morphology within clades. In the Leafy I clade, leaf insertion is strictly incubous or transverse (the latter only in *Lepidolaena* and *Jubulopsis*), the leaves are complicate-bilobed, underleaves are present (except in *Radula*), and perianths are typical, except in the *Gackstroemia* through *Goebeliella* group, where a coelocaule is elaborated. In this clade, many taxa have lobules that are shaped as water-sacs, but some produce flattened lobules.

In the Leafy IIA clade, most taxa have incubous or transverse insertion, well-developed underleaves, and multiple-lobed leaves. However, *Plagiochila* and *Chiloscyphus* have succubously inserted leaves that are generally entire or 2-lobed, and the underleaves may be reduced or absent.

In the Leafy IIB clade, leaf insertion is strictly succubous or transverse but leaf shape is variable; in this clade, most taxa have extremely reduced or absent underleaves (except *Tetralophozia*, with larger underleaves).

In the Leafy IIC clade, leaf insertion, shape, and underleaf development vary widely; however, in this clade are concentrated the taxa that form fleshy perigynia (e.g., *Isotachis*) and marsupia (e.g., *Calypogeia*). Taxa with marsupia are not found elsewhere in the phylogeny.

For the basal members of the Leafy II clade, insertion is incubous (*Neotrichocolea*, *Ptilidium*, and *Schistochila*) or transverse (*Temnoma*), leaves are multi-lobed (and *Neotrichocolea* has water sacs), underleaves are present and well developed, and a perianth (*Neotrichocolea*, *Ptilidium*) or coelocaule (*Schistochila*, *Temnoma*) is present.

NOVEL HYPOTHESES SUGGESTED BY THE PHYLOGENY

Perhaps the most striking result of these phylogenetic analyses is that *Pleurozia* appears to be more closely related to the Metzgeriaceae and Aneuraceae than to any of the leafy liverworts. This has not been previously hypothesized; because *Pleurozia* is leafy and has a complicated lobulate leaf, it has always been thought of as a derived taxon (Schuster, 1984; Crandall-Stotler & Stotler, 2000). It has always been classified in an isolated position, however, because of its unusual characteristics (Schuster, 1984; Crandall-Stotler & Stotler, 2000).

Pleurozia is composed of 11 species and distributed primarily in the montane tropics; however, *P. purpurea* Lindb. has an interrupted Northern Hemisphere distribution in areas with oceanic climates and high precipitation levels (Thiers, 1993; Schofield, 2002). All but one species (*P. paradoxa* (Jack) Schiffn.) possess lobules; however, they are dorsal in orientation (this was only recently discovered; the plants were previously described upside-down (Schuster, 1965; Thiers, 1993)). Among the liverworts, *Pleurozia* alone has saccate lobules that are dorsal, rather than ventral. Further, in many species the lobule is a completely enclosed water sac and has a complex hinged, spathulate valve at the lobule aperture (Thiers, 1993). The valve opens and closes according to the level of water-sac hydration (Crandall-Stotler, 1976). Such a mechanism is unique to liverworts. In addition, *Pleurozia* has a lenticular apical cell with only two cutting faces, while the rest of the core leafy liverworts have a tetrahedral apical cell with three cutting faces

(Crandall-Stotler, 1976). The lenticular apical cell is common among the simple thalloid liverworts, including the Metzgeriaceae and Aneuraceae (Crandall-Stotler & Stotler, 2000). This observation, along with the phylogeny, suggest that the tetrahedral apical cell is an invariant feature in the core leafy liverworts, and that the presence of a lenticular apical cell is a shared characteristic in the clade of simple thalloids including *Pleurozia*. Although the sequence of cell divisions is unique in *Pleurozia*, leaf development generally follows the pattern seen in the core leafy liverworts; that is, the leaf develops from two initials (Crandall-Stotler, 1976). This is in contrast to the semi-leafy simple thalloid liverworts, such as *Fossombronia*, where leaves develop from a single growing point (Crandall-Stotler, 1981).

The complicate-bilobed liverworts in the families Porellaceae (*Ascidiota*, *Porella*), Lejeuneaceae (*Bryopteris*, *Ceratolejeunea*, *Cylolejeunea*, *Lejeunea*, *Odontolejeunea*), Jubulaceae (*Frullania*, *Jubula*), and Radulaceae (*Radula*) are part of a clade that represents an early dichotomy in the leafy liverworts (Fig. 2, Leafy I). These taxa have always been placed at the end of any classification (Tables 7, 8), indicating positions as crown groups in the leafy liverworts. These contrasting hypotheses suggest that characteristics thought to be recently derived in these taxa (such as endosporic germination and the ventral lobule) may actually represent early innovations.

Neotrichocolea (Neotrichocoleaceae) and *Schistochila* (Schistochilaceae) also have ventral lobules. The placement of *Neotrichocolea* is somewhat ambiguous; it is sister to *Ptilidium* in all analyses of the full matrix (Fig. 2), and there is no significant difference between parsimony trees in the full analysis when it is resolved in the Leafy I versus Leafy II clades (as indicated by Templeton and SH tests). However, *Ptilidium* and *Neotrichocolea* are always resolved in the Leafy II clade under parsimony when the position of *Ptilidium* is unconstrained, as well as under homogeneous and heterogeneous Bayesian analyses. *Schistochila* is always resolved as an early lineage in Leafy II regardless of the placement of *Ptilidium*. Intriguingly, this suggests that the lobulate leaf may be the plesiomorphic state in the leafy liverworts. If this remains supported, hepaticologists may be required to completely reverse their thinking about morphological evolution of the leaf in this group. It appears, based on these analyses, that the prostrate, dorsiventral, and anisophyllous leafy gametophyte may have arisen earliest in the evolution of the group and erect, isophyllous taxa may have evolved later. In fact the Herbertaceae, placed near the beginning of any classification based on their supposedly primitive isophyllous, transversely inserted leaves (Tables 7, 8), occupy a derived position (Fig. 2, Leafy IIA).

THE DIRECTIONALITY OF LEAFY LIVERWORT EVOLUTION: WHAT IS PRIMITIVE?

Characteristics most commonly labeled as primitive in the leafy liverworts do not appear to be so, as interpreted in the context of the phylogenies presented here. Isophyllous or nearly isophyllous taxa appear in derived positions in the tree (e.g., *Herbertus*, *Triandrophyllum*, *Anthelia*; Figs. 1, 2); taxa with highly divided leaves and massive sporophytes are dispersed throughout the tree and sometimes appear in derived positions (e.g., *Trichocolea*, *Blepharostoma*, *Temnoma*; Figs. 1, 2). Further, taxa with supposedly recently advanced traits do not appear to be in crown positions (e.g., the Lejeuneaceae; Figs. 1, 2). The phylogeny suggests that one of these advanced traits, the lobule, may be plesiomorphic in the leafy liverworts. Other characters thought to be primitive (Shuster, 1984), such as fasciculate rhizoids, appear to be supported by the phylogeny. Formal character reconstructions are needed in order to interpret the polarity of such characters.

THE FIRST LIVERWORT—A SIMPLE THALLOID?

The simple thalloid liverworts are not monophyletic, and it appears that they may have been the earliest liverworts. This hypothesis has been advanced previously (Schofield, 1985) and is supported by fossil data (Hueber, 1961; reviewed in Schuster, 1966, and Krassilov & Schuster, 1984). The topology presented here is in complete agreement with a more detailed analysis of the simple thalloid liverworts (Forrest & Crandall-Stotler, 2004 cf. this volume).

Blasia, always classified previously with the simple thalloid liverworts, appears more closely related to the complex thalloid liverworts; this result is strongly supported in all analyses (BS = 100, posterior probability = 100%; Fig. 2). The single species, *Blasia pusilla* L., is distributed across the Northern Hemisphere. It has unique, flask-shaped gemma containers and is the only liverwort with symbiotic *Nostoc* colonies embedded in its thallus. Although *Blasia* is a simple thalloid in terms of gametophyte structure, it possesses ventral scales, a feature seen elsewhere only in marchantioids (Renzaglia, 1982). Pass and Renzaglia (1995) recommended that *Blasia* be grouped with the mar-

Table 7. Classification of the Jungermanniales fide Schuster (1984) and genera sampled within each taxon. Numbers in parentheses indicate number of species samples, if other than one.

Suborder	Family	Genera sampled	No. of genera in family
Herbertineae	Herbertaceae	*Herbertus* (2 spp.), *Triandrophyllum*	2
	Trichocoleaceae	*Trichocolea, Blepharostoma, Temnoma*	13
	Grolleaceae	—	1
	Trichotemnomataceae	—	1
Antheliineae	Antheliaceae	*Anthelia*	1
Lepicoleineae	Vetaformaceae	—	1
	Lepicoleaceae	*Lepicolea* (2)	1
Jungermanniineae	Jungermanniaceae	*Jamesoniella, Jungermannia* (2), *Lophozia, Nardia, Tetralophozia, Tritomaria, Stenorrhipis*	32
	Gymnomitriaceae	*Gymnomitrion, Herzogobryum, Marsupella*	7
	Scapaniaceae	*Diplophyllum, Scapania*	2
	Blepharidophyllaceae	—	3
	Delavayellaceae	—	1
Geocalycineae	Geocalycaceae	*Chiloscyphus, Harpanthus, Lophocolea*	21–23
	Plagiochilaceae	*Plagiochila*	9–10
	Acrobolbaceae	*Marsupidium, Lethocolea*	6
	Arnelliaceae	—	3
Brevianthineae	Brevianthaceae	—	1
	Chonecoleaceae	—	1
Perssoniellineae	Schistochilaceae	*Schistochila*	4
	Perssoniellaceae	—	1
Balantiopsidineae	Balantiopsidaceae	*Balantiopsis, Isotachis*	6
	Gyrothyraceae	—	1
Lepidoziineae	Lepidoziaceae	*Acromastigum, Bazzania, Lepidozia, Teleranea*	28
	Phycolepidoziaceae	—	1
	Calypogeiaceae	*Calypogeia*	2
Cephaloziineae	Cephaloziaceae	*Cephalozia, Nowellia, Odontoshisma*	12
	Cephaloziellaceae	*Cephaloziella*	7
	Jackiellaceae	—	1
	Adelanthaceae	*Adelanthus*	2
Ptilidiineae	Ptilidiaceae	*Ptilidium*	1
	Mastigophoraceae	*Mastigophora*	1
	Caetophyllopsidaceae	—	2
Lepidolaenineae	Lepidolaenaceae	*Gackstroemia, Lepidolaena*	3
	Jubulopsidaceae	*Jubulopsis*	1
	Neotrichocoleaceae	*Neotrichocolea*	2
Porellineae	Goebeliellaceae	*Goebeliella*	1
	Porellaceae	*Ascidiota, Porella*	3
	Jubulaceae	*Frullania, Jubula*	5
	Lejeuneaceae	*Bryopteris, Ceratolejeunea, Cyclolejeunea, Lejeunea, Odontolejeunea*	75
Radulineae	Radulaceae	*Radula* (2)	1
Pleuroziineae	Pleuroziaceae	*Pleurozia*	1

chantioids, and these analyses support that conclusion. Moreover, its position as the first branch in the complex thalloid clade suggests that characters typifying the marchantioids (e.g., four primary androgonial cells, unistratose capsule walls, and spore mother cells that are unlobed) are derived from a simple thalloid type (alternatively, these features could have been lost or represent reversals in *Blasia*).

Likewise, features shared between *Haplomitrium* and the simple thalloid liverworts appear to be plesiomorphic for the liverworts as a whole. Such features may include whip-like elaters, lack of wall pigments, central conducting strand in the axis, and

Table 8. Classification of the Jungermanniideae fide Crandall-Stotler and Stotler (2000) and genera sampled within each taxon. Numbers in parentheses indicate number of species samples, if other than one.

Order	Suborder	Family	Genera sampled	No. of genera in family
Lepicoleales	Ptilidiineae	Ptilidiaceae	*Ptilidium*	1
		Mastigophoraceae	*Mastigophora*	2
		Chaetophyllopsidaceae	—	2
	Lepicolieneae	Vetaformataceae	—	1
		Lepicoleaceae	*Lepicolea* (2 spp.)	1
	Perssoniellineae	Schistochilaceae	*Schistochila*	5
		Perssoniellaceae	—	1
	Lepidolaenineae	Trichocoleaceae	*Trichocolea*	2
		Lepidolaenaceae	*Gackstroemia, Lepidolaena*	3
		Neotrichocoleaceae	*Neotrichocolea*	1
		Jubulopsidaceae	*Jubulopsis*	1
Jungermanniales	Herbertineae	Herbertaceae	*Herbertus* (2), *Triandrophyllum*	2
		Pseudolepicoleaceae	*Blepharostoma, Temnoma*	7
		Trichotemnomataceae	—	1
		Grolleaceae	—	1
	Balantiopsidineae	Balantiopsidaceae	*Balantiopsis, Isotachis*	8
	Lophocoleineae	Geocalycaceae	*Chiloscyphus, Harpanthus, Lophocolea*	25
		Gyrothraceae	—	1
		Plagiochliaceae	*Plagiocila*	8
		Acrobolbaceae	*Marsupidium, Lethocolea*	7
		Arnelliaceae	—	3
	Lepidoziineae	Phycolepdoziaceae	—	1
		Calypogeiaceae	*Calypogeia*	3
		Lepidoziaceae	*Acromastigum, Bazzania, Lepidozia, Telaranea*	27
	Cephaloziineae	Cephaloziaceae	*Cephalozia, Nowellia, Odontoshisma*	15
		Cephaloziellaceae	*Cephaloziella, Stenorrhipis*	9
		Jackiellaceae	—	1
		Adelanthaceae	*Adelanthus*	2
	Antheliineae	Antheliaceae	*Anthelia*	1
	Brevianthineae	Brevianthaceae	—	1
		Chonocoleaceae	—	1
	Jungermanniineae	Jungermanniaceae	*Jamesoniella, Jungermannia* (2), *Lophozia, Nardia, Tetralophozia, Tritomaria*	37
		Mesoptychiaceae	—	1
		Gymnomitriaceae	*Gymnomitrion, Herzogobryum, Marsupella*	13
		Scapaniaceae	*Diplophyllum, Scapania*	8
Porellales	Porellineae	Porellaceae	*Ascidiota, Porella*	3
	Jubulineae	Geobeliellaceae	*Goebeliella*	1
		Jubulaceae	*Frullania, Jubula*	2
		Bryopteridaceae	*Bryopteris*	1
		Lejeuneaceae	*Ceratolejeunea, Cyclolejeunea, Lejeunea, Odontolejeunea*	92
Radulales		Radulaceae	*Radula* (2)	1
Pleuroziales		Pleuroziaceae	*Pleurozia*	1

fleshy shoot calyptra (Schuster, 1984), as well as leaf development from only one initial (Crandall-Stotler, 1981). In addition, monoplastidic meiosis, found only in *Blasia, Haplomitrium,* and *Monoclea* among liverworts (Renzaglia et al., 1994), also appears to be a liverwort plesiomorphy.

An unambiguous phylogenetic placement for *Haplomitrium* remains elusive. Although in most

analyses it was resolved as sister to the simple thalloids plus the leafy clades, in agreement with the initial dichotomy hypothesis, this did not appear in parsimony analyses of the full matrix, or 21-model analyses of the backbone data matrix. However, most evidence supports *Haplomitrium* as sister to the Jungermanniopsida. The placement of this taxon was also equivocal in more detailed maximum parsimony and maximum likelihood analyses of simple thalloid liverworts (Forrest & Crandall-Stotler, 2004 cf. this volume). These analyses clearly support the hypothesis that *Haplomitrium* is a unique evolutionary line, not closely related to any of the three main lineages of liverworts. It is interesting to note that molecular data are nearly as difficult to interpret as the morphological data in this extremely isolated group; the long branches caused by molecular apomorphies appear to be correlated with the accumulation of morphological apomorphies. Further analysis is needed to more confidently identify its phylogenetic position. The phylogenetic position of *Haplomitrium* is important for interpreting the polarity of characteristics, such as apical cell shape, growth habit, and the presence of leaves. Character reconstructions on the phylogeny, using methods that can take phylogenetic uncertainty as well as branch length into consideration, are needed.

The results of these analyses bring us no further toward visualizing whether the first liverwort was leafy and erect or thallose and prostrate. However, it seems clear that the leafy and simple thalloid morphologies have been evolutionarily reinvented multiple times. For instance, many genera of simple thalloid liverworts are somewhat leafy (e.g., *Phyllothallia, Noteroclada*), and some groups of leafy liverworts apparently have secondarily evolved a more thallose shape (e.g., *Metzgeriopsis* and *Zoopsis* (Lepidoziaceae), *Schiffneria* (Cephaloziaceae)). Perhaps the phylogenetic placement of *Pleurozia* most clearly illustrates how labile these characteristics appear to be.

Possible Missing Lineages in the Phylogeny

Although this research effort represents a considerable step toward understanding liverwort phylogeny, several unsampled groups need consideration in future work. For instance, *Treubia*, a unique taxon usually classified in or near the simple thalloids (Schuster, 1984; Crandall-Stotler & Stotler, 2000), is semi-leafy and has numerous unique characteristics that call its phylogenetic placement into question. The presence of *Treubia* in analyses of simple thalloid phylogeny drastically affects the placement of *Haplomitrium*. When included, the two taxa are sisters and resolved as the first branching lineage of liverworts; however, when *Treubia* is excluded, *Haplomitrium* is resolved within the simple thalloids (Forrest & Crandall-Stotler, 2004 cf. this volume).

The phylogenetically isolated positions of *Ptilidium*, *Neotrichocolea*, *Schistochila*, and *Temnoma* in the Leafy II clade, along with uncertainty associated with the placement of *Ptilidium*, suggest that additional sampling among related taxa in this area of the tree is needed. This is highlighted by the fact that their taxonomically hypothesized relatives appear in completely different areas of the tree. Although there is no way to determine a priori which taxa may appear in these phylogenetic positions, additional samples from the Schistochilaceae and Chaetophyllopsidaceae, as well as *Perssoniella*, *Trichotemnoma*, and *Grollea*, are suggested. Further sampling among these taxa may also allow a more definitive interpretation of the position of *Ptilidum* and *Neotrichocolea*, thus giving a clearer picture of the evolution of the ventral lobule. Finally, *Adelanthus* is not always supported as part of the Leafy IIB clade and falls into an unresolved position (see Results), indicating that additional sampling in the genus or family (Adelanthaceae) may be needed.

In addition to further sampling to resolve relationships at the subordinal level, the results of these analyses suggest problematic areas requiring further examination at the family or genus rank. For instance, the Jungermanniaceae, Gymnomitriaceae, Geocalycaceae, and Cephaloziellaceae appear to be a complex of interdigitated families. Surprisingly, the genera *Lophocolea* and *Chiloscyphus*, sometimes included in a single genus (Geocalycaceae), appear to be unrelated based on the analyses presented here.

Conclusion

Should we reclassify the liverworts based on the results of these analyses? If, upon careful examination of the phylogeny in conjunction with morphology, useful characteristics are recognized that can be used to define clades, it should be done. Character reconstruction analyses may be a first step toward this end. However, it is clear that this ancient plant lineage is rife with morphological homoplasy, and characters which formerly united clades may have been forever obscured by the processes of evolution and extinction. More important than a guideline for reclassification, this research will allow evolutionary biologists to examine ques-

tions about hepatics within a testable evolutionary framework.

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PHYLOGENY OF LIVERWORTS—BEYOND A LEAF AND A THALLUS[1]

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Abstract

A reconstruction of phylogenetic relationships of the liverworts is presented using three chloroplast genome *rbc*L, *rps*4, and *trn*L-F regions obtained for 123 plant species. These include 99 liverworts (in 46 families and 76 genera), two hornworts, 13 mosses, six vascular plants, and three green algae. Analyses based on the maximum parsimony principle using both direct optimization (POY) and also static alignment (Nona) were done. The data sets were analyzed separately as well as simultaneously with different combinations of parameter values. Our study suggests that liverworts compose a monophyletic group representing the earliest lineage of land plants. *Blasia pusilla* is supported to form its own class, Blasiopsida, and it is the sister group of the rest of the liverwort species. We also suggest that the ancestral type of the liverworts is likely to have been a simple thalloid gametophyte, with growth via a cuneate apical cell with four cutting faces, bearing a sporophyte with massive foot and seta. The complex thalloid liverworts are monophyletic, and a sister group to the simple thalloid and leafy liverworts. The complex thalloid and simple thalloid/leafy liverworts represent distinct lineages, and this is a well-supported result. The simple thalloid liverworts are redefined to remove the acrogynous taxa to the jungermannioid leafy liverwort group, and the simple thalloid liverworts constitute a monophyletic group. The leafy liverworts are resolved as a monophyletic group composed of two sister clades, the *Pleurozia–Noteroclada–Metzgeria* clade and a second clade that is further divided into two sister groups, a Porellales–Lepidolaenineae clade and a Perssoniellineae–Herbertineae clade. The sister relationship between *Pleurozia* and *Noteroclada* and *Metzgeria* suggests that separating the leafy and simple thalloid liverworts by only leaf initial criterion is probably not feasible. Our results suggest that the bilateral and anisophyllous gametophyte organization and branching from only lateral merophytes that is mostly present in the Porellales–Lepidolaenineae clade are ancestral, whereas the triradial and isophyllous organization and branching from both lateral and ventral merophytes of most of the Perssoniellineae–Herbertineae clade are derived states. The leafy liverworts, which have branching from both lateral and ventral merophytes, have evolved into three evolutionary lines: the *Blepharostoma–Isotachis* clade, the Jungermanniineae–Cephaloziineae clade, and the Lepidoziineae–Geocalycineae–Lepicoleineae–Herbertineae clade.

Key words: bryophytes, chloroplast genome, classification, direct optimization, evolution, hepatics, liverworts, parsimony analysis, phylogeny, *rbc*L, *rps*4, *trn*L-F.

Extant bryophytes, which comprise the liverworts, hornworts, and mosses, have been assumed to be the closest living relatives of the first land plants in various studies (Mishler et al., 1994; Kenrick & Crane, 1997a, b). They retain various supposedly primitive features, such as unbranched sporophytes attached to the gametophytes, dependence on water for fertilization, and simple vascular tissue. They also share characters with the other embryophytes in having multicellular sporophytes, archegonia, antheridia, and a cuticle. Therefore, phylogenetic studies of bryophytes should enhance our understanding of land plant origin, evolution, and diversity.

Liverworts have been much less investigated with molecular markers than mosses. Fundamental questions as to whether the liverworts are a monophyletic group, and where their systematic position is among the land plants, are not fully answered. Phylogenetic relationships among liverworts remain ambiguous at different levels. Comprehensive phylogenetic studies on the leafy liverworts, which rep-

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resent 85% of liverwort species diversity (Schuster, 1984a), are still lacking.

Since the liverworts as a plant group was established in 1789 by A. L. de Jussieu, the direction in which the liverwort evolution has proceeded has remained controversial. The long-standing opinion on this issue is given by Schuster (1966, 1972, 1979, 1984a, 2000, 2002), who proposed that the gametophytes of the ancestral type were erect, radial, terminated by a tetrahedral apical cell, and that the sporophytes were well developed and chlorophyllous. However, Schuster's hypotheses were not formally tested until Mishler and Churchill (1984, 1985) made a formal cladistic analysis on liverworts with 24 morphological characters, in which the complex thalloid *Sphaerocarpos* Boehm. was resolved as sister to the rest of the liverworts.

Recently, phylogenetic studies on liverworts using molecular data have been mostly focused on relationships of bryophytes and other land plants (e.g., Kranz et al., 1995; Duff & Nickrent, 1999). Studies within the liverworts to date have mostly been based each on a single genomic region and limited taxon selection (e.g., Capesius & Bopp, 1997; Stech & Frey, 2001). Clearly, results compiled from the above studies need to be further tested by using multiple gene data together with morphology, and sufficient numbers of taxa should be applied to reconstruct phylogenetic relationships of the liverworts.

To explore phylogenetic relationships of liverworts to other land plants, and among liverworts, especially within different leafy liverwort lineages, we compared molecular data of 123 species representing green algae and all major land plant lineages. The ingroup of the study included 99 species of liverworts, 78 of them leafy. We used DNA sequences from chloroplast gene regions *rbc*L, *trn*L-F, and *rps*4. The phylogenies were constructed with cladistic methods using direct optimization (POY) and also parsimony analysis based on static alignment (Nona).

Historical Outline

Liverwort nomenclature begins with *Species Plantarum* of Linnaeus (1753). One of the first comprehensive classifications based on morphological criteria was proposed by Endlicher (1841). Endlicher assumed that liverworts with simple-structured sporophytes were primitive, so he put the thalloid liverworts, like *Riccia* L., at the beginning and the leafy ones at the end in his system. This is in agreement with the antithetic theory of sporophyte origins (Celakovsky, 1874; Bower, 1890). Schiffner (1893–1895) followed the same arrangement of higher ranks, but further established the categories Jungermanniales akrogynae (leafy liverworts) and Jungermanniales anakrogynae (simple thalloid liverworts). The segregation of leafy and simple thalloid liverworts was initiated from Leitgeb's (1874–1881) theory of archegonial formation, in which acrogyny and anacrogyny were defined so that in acrogyny the formation of archegonia terminates further apical cell segmentation (as in the leafy liverworts); whereas in anacrogyny, formation of archegonia does not terminate the apical cell segmentation (as in simple thalloid liverworts). Wettstein (1903–1908) proposed the opposite, reductive course of evolution. According to Wettstein, the erect, radial, leafy gametophytes were primitive, and the thalloid liverworts were derived. Wettstein's system was followed by Verdoorn (1932), Buch (1936), and later developed by Evans (1939).

In the contemporary systematic studies on liverworts, efforts, starting with Schuster (1953), have been made to sort out familial and generic boundaries, and to create a sounder phylogenetic sequence at different taxonomic levels. Consequently, 15 new families had been created in order to solve the problems of heterogeneous families (Schuster, 1955, 1957, 1960, 1963, 1967, 1972, 1984b; Fulford & Taylor, 1963; Grolle, 1972), and many suborders, such as Lepicoleineae R. M. Schust., Lepidolaenineae R. M. Schust., Balantiopsidineae R. M. Schust., Perssoniellineae R. M. Schust., Cephaloziineae R. M. Schust., and Geocalycinae R. M. Schust., were described (Schuster 1972). Although Schuster (1966, 1972) proposed his systems based on his studies of a wide species distribution range, including tropical and antipodal regions, relationships among certain groups of the liverworts remained unresolved. Schljakov (1972) elevated Schuster's suborders as orders and orders as superorders, and presented minor changes on family arrangements.

Schuster stated that liverworts and mosses share a common ancestral type, and the closest extant plant would be the liverwort *Haplomitrium* Nees (Schuster 1958, 1966, 1972, 1979, 1984a, 2000). He suggested that the initial dichotomy in liverworts was between the complex thalloid liverworts and rest of the liverworts (Schuster, 1984a). Moreover, Schuster (1979, 2000, 2002) assumed a bush-like phylogeny for the leafy liverworts and hypothesized that the same pattern of morphological evolution was repeated in different leafy liverwort suborders, an evolution from erect and isophyllous to creeping and distinctively anisophyllous plants. Schuster's system consists of seven orders, in which

the leafy liverworts (Jungermanniales) are divided in 15 suborders and the simple thalloids (Metzgeriales) in seven suborders. Considering the ranking of liverworts, Schuster (2000) wished to preserve the conservative classification of order Jungermanniales. In the latest liverwort classification, based mainly on cladistic analysis with morphological characters, Crandall-Stotler and Stotler (2000) described a liverwort phylum Marchantiophyta, which were divided into the two classes Marchantiopsida Stotler & Stotl.-Crand. and Jungermanniopsida Stotler & Stotl.-Crand. The latter consisted of subclasses Metzgeriidae Barthol.-Began and Jungermanniidae Stotler & Stotl.-Crand. Crandall-Stotler and Stotler (2000) placed *Haplomitrium*, *Blasia* L., and *Treubia* K. I. Goebel at the beginning of Metzgeriidae, but they argued that their natural placements cannot be solved with confidence by using morphological characters alone. Phylogenetic inferences based on these characters resolved the leafy liverworts, or Jungermanniidae, as a crown group of Jungermanniopsida and, hence, the Metzgeriidae as a paraphyletic group. In the absence of comprehensive phylogenetic analyses of leafy liverworts, Crandall-Stotler and Stotler (2000) relied on Schuster's (1972, 1984a) work when developing their own classification (see Table 1).

Evidence from Phylogenetic Studies

Inferences from various sources of characters, i.e., morphology, ontogeny, ultrastructure, DNA sequences, and gene structure, have failed to converge on a single phylogenetic hypothesis for land plant evolution (see Goffinet, 2000). Monophyly of the liverworts has also been controversial in phylogenetic studies. Various studies of bryophyte 18S rRNA data resolved liverworts as paraphyletic (Capesius, 1995; Bopp & Capesius, 1996, 1998; Capesius & Bopp, 1997). On the other hand, Hedderson et al. (1996, 1998) resolved the liverworts as a monophyletic group based on the same locus but with different taxa. Furthermore, studies with a variety of other molecular markers from nuclear (Mishler et al., 1992), chloroplast (Lewis et al., 1997; Stech & Frey, 2001), and mitochondrial (Qiu et al., 1998; Beckert et al., 1999) genomes as well as analyses with morphological (Crandall-Stotler & Stotler, 2000), ultrastructural, and developmental characters (Garbary et al., 1993; Garbary & Renzaglia, 1998) support the monophyly of the liverworts.

In most phylogenetic studies, the complex thalloid liverworts and the rest of the liverworts are distinctive groups (e.g., Bopp & Capesius, 1996, 1998; Hedderson et al., 1996, 1998; Capesius & Bopp, 1997; Lewis et al., 1997; Beckert et al., 1999; Crandall-Stotler & Stotler, 2000; Wheeler, 2000). Stech et al. (2000) and Stech and Frey (2001) found two more lineages, Treubiopsida Stech, J.-P. Frahm, Hilger & W. Frey and Blasiopsida Stech & W. Frey, using *trn*L intron data. They rejected the initial dichotomy of complex thalloid liverworts versus the rest of the liverworts.

Simple thalloid and leafy liverworts have not been resolved as monophyletic groups in previous phylogenetic analyses. *Metzgeria* Raddi fell within the leafy liverworts in some *rbc*L (Lewis et al., 1997) and in the *nad*5 analyses (Beckert et al., 1999). The recent morphological cladistic analysis by Crandall-Stotler and Stotler (2000) showed the simple thalloid liverworts to be a paraphyletic group. Stech and Frey (2001) stated that there have been too few taxa included in most analyses to conclude anything about differences between those groups. Their own *trn*L intron data analyses indicated that simple thalloid liverworts evolved independently in different evolutionary lineages, which belong to different liverwort classes in their classification (Stech & Frey, 2001). So far, no DNA evidence has been used to extensively address relationships among leafy liverworts.

Materials and Methods

TAXON SAMPLING

The exemplars represent all the main taxonomic lineages of land plants, with special emphasis on the liverworts, and especially the leafy liverworts. Samples other than liverworts were considered outgroup species, including three green algae. The sample and voucher information is given in Table 1. The shoots were sampled from material under a dissecting microscope. When possible, only the youngest parts of the shoots were taken, but for very small plants, whole shoots were used. As many species are extremely small, it was often necessary to use several shoots or individuals. These were always sampled from the same specimen. The most recent specimens used in the present study are from the year 2001, and the oldest is from 1976 (*Vetaforma dusenii*).

MOLECULAR MARKERS

We used three chloroplast genes, *rbc*L, *trn*L-F, and *rps*4, as molecular markers. GenBank accession numbers for the sequences are given in Table 1. The percentage of missing data was about 12% across all the three genic regions. The percentage

Table 1. Taxa and the GenBank accession numbers for the sequences used in the present study. The accession numbers for sequences taken from the GenBank are shown in italics. The accession numbers published in Ahonen (2004) contemporarily in this same volume are marked with asterisks. The voucher specimens for novel sequences herein, as well as references for sequences taken from the GenBank are listed under vouchers. Liverworts are arranged to orders and suborders according to Crandall-Stotler and Stotler (2000) classification.

Taxon	GenBank accession No.			Voucher
	*rbc*L	*rps*4	*trn*L-F	
LIVERWORTS				
Class Marchantiopsida				
Order Sphaerocarpales, suborder Sphaerocarpineae				
Sphaerocarpos texanus Austin	*U87090*			Lewis et al. (1997)
		AF231891	*AF231901*	Newton et al. (2000)
Order Monocleales				
Monoclea gottschei Lindb.	*U87083*			Lewis et al. (1997)
		AJ251063		Capesius & Bloecher (unpublished)
			AF228788	Wheeler (2000)
Order Marchantiales, suborder Marchantiineae				
Reboulia hemisphaerica (L.) Raddi	AY462326		AY463584	Japan, *Itouga & Mori* (H)
Wiesnerella sp. ined. 1	AY462336	AY462402	AY463594	China, *He-Nygrén 777*
Conocephalum conicum (L.) Underw.	*U87067*			Lewis et al. (1997)
			AF071834	Meissner et al. (1998)
Lunularia cruciata (L.) Dumort. ex Lindb.	*U87077*			Lewis et al. (1997)
			AF228790	Wheeler (2000)
Marchantia polymorpha L.	*U87079*			Lewis et al. (1997)
		X04465		Yamano et al. (1984)
			AF228786	Wheeler (2000)
Order Ricciales				
Ricciocarpos natans (L.) Corda	*U87090*			Lewis et al. (1997)
		AJ251062		Capesius & Bloecher (unpublished)
			AF227672	Wheeler (2000)
Class Jungermanniopsida				
Order Haplomitriales				
Haplomitirum mnioides (Lindb.) R. M. Schust.	*U87071*			Lewis et al. (1997)
		AF231890	*AF231900*	Newton et al. (2000)
Haplomitrium hookeri (Sm.) Nees	*U87072*			Lewis et al. (1997)
		AJ251064		Capesius & Bloecher (unpublished)
			AY007642	Stech & Frey (2001)

Table 1. Continued.

Taxon	GenBank accession no. *rbc*L	*rps*4	*trn*L-F	Voucher
Order Blasiales				
Blasia pusilla L.	AY462288	AY462342	AY463547	Finland, *Thurén 2766* (H)
Order Fossombroniales, suborder Fossombroniineae				
Fossombronia foveolata Lindb.	*U87069*			Lewis et al. (1997)
			AY007620	Stech & Frey (2001)
Suborder Pelliineae				
Noteroclada confluens Taylor ex Hook. f. & Wilson	AY462318	AY462377	AY463577	Argentina, *Norris 97836* (H)
Pellia epiphylla (L.) Corda	*U87085*			Lewis et al. (1997)
			AF071842	Meissner et al. (1998)
Pellia neesiana (Gottsche) Limpr.		*AJ250456*		Capesius & Bloecher (unpublished)
			AF180683	Pacak et al. (2002)
Pellia sp. ined. 1	AY462323	AY462383		China, *Koponen et al. 47823*
Order Metzgeriales, suborder Pallaviciniineae				
Pallavicinia sp. ined. 1		AY462380	AY463580	China, *He-Nygrén 1407*
Makinoa sp. ined. 1	AY462313	AY462370	AY463573	China, *He-Nygrén 1295*
Hymenophyton flabellatum (Labill.) Dumort. ex Trevis.		AY462357		Australia, *Streimann 16733* (H)
			AF071840	Meissner et al. (1998)
Suborder Metzgeriineae				
Metzgeria conjugata Lindb.	AY462315	AY462373		Slovakia, *Huttunen & Jalkanen 191* (H)
			AY007625	Stech & Frey (2001)
Metzgeria furcata (L.) Corda	*U87081*			Lewis et al. (1997)
			AY007626	Stech & Frey (2001)
Order Lepicoleales, suborder Ptilidiineae				
Ptilidium pulcherrimum (F. Weber) Hampe	AY302460*		AY251186*	Ahonen (2004)
		AY462388		Finland, *Ahonen & Engblom 7*
Dendromastigophora flagellifera (Hook.) R. M. Schust.	AY462294		AY463555	New Zealand, 2001, *Glenny 8520*
Mastigophora woodsii (Hook.) Nees	AY462314	AY462372	AY463574	Australia, *Frahm* (CANB 639918)
Chaetophyllopsis whiteleggei (Carrington & Pearson) R. M. Schust. ex Hamlin	AY462292	AY462346	AY463553	Australia, *Curnow 4804* (H)

Table 1. Continued.

Taxon	GenBank accession no.			Voucher
	*rbc*L	*rps*4	*trn*L-F	
Suborder Lepicoleineae				
Vetaforma dusenii (Steph.) Fulford & J. Taylor	AY462335		AY463593	Chile, *Engel 11423* (H)
Lepicolea ochroleuca (L. f. ex Spreng.) Spruce	AY462306	AY462364	AY463566	Chile, *Hyvönen 2938* (H)
Lepicolea pruinosa (Taylor) Spruce	AY462307		AY463567	Peru, *Frahm et al. s.n.* (H)
Lepicolea scolopendra (Hook.) Dumort. ex Trevis.	AY462308	AY462365	AY463568	Australia, *Streimann 55445* (H)
Suborder Perssoniellineae				
Gottschea nuda (Horik.) Grolle & Zijlstra	AY462297	AY462351	AY463558	Japan, *Yamaguchi* (H)
Paraschistochila tuloides (Hook. f. & Taylor) R. M. Schust.	AY462321	AY462381	AY463581	New Zealand, 2001 *Glenny 8499*
Schistochila appendiculata (Hook.) Dumort. ex Trevis.	AY462328	AY462394	AY463596	New Zealand, 2001, *Glenny 8537*
Schistochila laminigera (Hook. f. & Taylor) A. Evans	AY462329	AY462395	AY463586	Chile, *Hyvönen 5888* (H)
Suborder Lepidolaenineae				
Trichocolea tomentella (Ehrh.) Dumort.	AY462332	AY462398	AY463590	China, *He-Nygrén 1137*
Leiomitra lanata (Hook.) R. M. Schust.	AY462305	AY462362	AY463565	New Zealand, 2001, *Glenny 8521*
Gackstroemia magellanica (Lam.) Trevis.	AY462295	AY462350	AY463556	Argentina, *Hyvönen 3079* (H)
Lepidogyna hodgsoniae (Grolle) R. M. Schust.		AY462366	AY463569	New Zealand, 2001, *Glenny 8500*
Lepidolaena clavigera (Hook.) Dumort. ex Trevis.	AY462309	AY462367		New Zealand, *Curnow 5079* (H)
Lepidolaena taylorii (Gottsche) Trevis.	AY462310	AY462368	AY463570	New Zealand, *Curnow 3554* (H)
Neotrichocolea bisseti (Mitt.) S. Hatt.	AY462317	AY462375	AY463576	Japan, *Inoue 5/03 200* (H)
Jubulopsis novae-zealandiae (E. A. Hodgs. & S. W. Arnell) R. M. Schust.	AY462304	AY462360	AY463564	New Zealand, 2001, *Glenny 8521*
Order Jungermanniales, suborder Herbertineae				
Herbertus sendtneri Steph.	AY462299	AY462353		Bhutan, *Long 28864* (H)
Herbertus dicranus (Taylor ex Gottsche) Trevis.	AY462300	AY462354	AY463559	Nepal, *Long 17542* (H)
Triandrophyllum subtrifidum (Hook. & Taylor) Fulford & Hatcher	AY462331	AY462397	AY463589	Chile, *Hyvönen 5334* (H)
Blepharostoma trichophyllum (L.) Dumort.	AY462289	AY462343	AY463548	Finland, *He-Nygrén & Piippo 1471*
Temnoma pilosum (A. Evans) R. M. Schust.	AY462330	AY462396	AY463588	Chile, *Hyvönen 5084*
Trichotemnoma corrugatum (Steph.) R. M. Schust.	AY462333	AY462399	AY463591	New Zealand, 2001, *Glenny 8426*
Suborder Balantiopsidineae				
Balantiopsis cancellata (Nees) Steph.	AY462286	AY462340	AY463545	Chile, *Hyvönen 5946* (H)
Isotachis armata (Nees) Gottsche		AY462358	AY463561	Papua New Guinea, *Koponen 32928* (H)
Isotachis humectata (Hook. f. & Taylor) Steph.	AY462302	AY462359	AY463561	Chile, *Hyvönen 5180* (H)

Table 1. Continued.

Taxon	GenBank accession no. *rbc*L	*rps*4	*trn*L-F	Voucher
Suborder Lophocoleineae				
Chiloscyphus cuspidatus (Nees) J. J. Engel & R. M. Schust.	*AY149845*		*AY149866*	He-Nygrén & Piippo (2003)
		AY462348		China, *Koponen et al. 48430*
Chiloscyphus minor (Nees) J. J. Engel & R. M. Schust.	*AY149843*	*AY149864*		He-Nygrén & Piippo (2003)
Chiloscyphus profundus (Nees) J. J. Engel & R. M. Schust.	*AY149852*		*AY149874*	He-Nygrén & Piippo (2003)
Heteroscyphus argutus (Reinw. & Nees) Schiffn.		AY462355		Nepal, *Long 30333* (H)
			AY149861	He-Nygrén & Piippo (2003)
Pedinophyllum truncatum Inoue	AY149855		*AY149878*	He-Nygrén & Piippo (2003)
		AY462382		China, *Koponen et al. 4678* (H)
Plagiochila asplenioides (L.) Dumort.	AY149839		*AY149858*	He-Nygrén & Piippo (2003)
		AY462384		Finland, *He-Nygrén & Piippo 1467*
Suborder Lepidoziineae				
Calypogeia integristipula Steph.	AY462290	AY462344	AY463550	Finland, *He-Nygrén & Piippo 1472*
Calypogeia muelleriana (Schiffn.) K. Müll.	*U87065*			Lewis et al. (1997)
			AY463551	Canada, *Ireland 21990* (H)
Bazzania sp. inded. 1	AY462287	AY462341	AY463546	China, *He-Nygrén 569*
Hygrolembidium acrocladum (Berggr.) R. M. Schust.	AY462301	AY462356	AY463560	Australia, *Streimann 16733* (H)
Lepidozia sp. ined. 1	AY462311		AY463571	China, *Koponen et al. 50623*
Zoopsis argentea (Hook. f. & Taylor) Gottsche, Lindenb. & Nees	AY462337	AY462403	AY463595	*Australia, Streimann 51704* (H)
Suborder Cephaloziineae				
Cephalozia bicuspidata (L.) Dumort.	AY462291	AY462345	AY463552	Finland, *Piippo 5655* (H)
Nowellia sp. ined. 1	AY462319	AY462378	AY463578	China, *He-Nygrén 1097*
Odontoschistoma sp. ined. 1	AY462320	AY462379	AY463579	China, *He-Nygrén 702*
Schiffneria hyalina Steph.	AY462327	AY462393	AY463585	Japan, *Mizutani 15961* (H)
Adelanthus lindenbergianus (Lehm.) Mitt.	AY462285		AY463544	Argentina, *Hyvönen 5637a* (H)
Suborder Jungermanniineae				
Chandonanthus sp. ined. 1	AY462293	AY462347	AY463554	China, *He-Nygrén 492*
Jamesoniella autumnalis (DC.) Steph.	AY462303		AY463563	China, *Koponen et al. 55912*
		AJ251066		Capesius & Bloecher, unpubl.
Jungermannia leiantha Grolle	AY149838		*AY149857*	He-Nygrén & Piippo (2003)

Table 1. Continued.

Taxon	GenBank accession no. *rbc*L	*rps*4	*trn*L-F	Voucher
		AY462361		Finland, *He-Nygrén & Piippo 1466*
Lophozia ventricosa (Dicks.) Dumort.	AY462312	AY462369	AY463572	Finland, *He-Nygrén & Piippo 1473*
Nardia assamica (Mitt.) Amakawa	AY462316	AY462374	AY463575	China, *Koponen et al. 49396*
Tritomaria quinquedentata (Huds.) H. Buch	AY462334	AY462400	AY463592	Finland, *He-Nygrén & Piippo 1474*
Gymnomitrion sp. ined. 1		AY462352	AY466440	Finland, *Parnela* (H)
Scapania undulata (L.) Dumort.	*AY149840*		*AY149859*	He-Nygrén & Piippo (2003)
		AY462392		Finland, *He-Nygrén & Piippo 1468*
Order Porellales, suborder Porellineae				
Porella japonica (Sande Lac.) Mitt.	AY302459*		AY251183*	Ahonen (2004)
Porella platyphylla (L.) Pfeiff.	AY302458*		AY251184*	Ahonen (2004)
		AY462387		Finland, *Ahonen & Engblom 28*
Suborder Jubulineae				
Goebeliella cornigera (Mitt.) Steph.	AY462296		AY463557	New Zealand, 2001, *Glenny*
Frullania dilatata (L.) Dumort.	*AY125929*		*AY144469*	Ahonen et al. (2003)
Frullania tamarisci (L.) Dumort.	AY302453*		AY251179*	Ahonen (2004)
		AY462349		Taiwan, *Ahonen, Huttunen & Virtanen 3187*
Jubula hutchinsiae (Hook.) Dumort.	*AY125946*		*AY144477*	Ahonen et al. (2003)
Jubula japonica Steph.	*AY125938*		*AY144479*	Ahonen et al. (2003)
Bryopteris filicina (Sw.) Nees	AY550110		AY463549	*Buck 14736* (H)
Aphanolejeunea gracilis Ast	AY302443*		AY251169*	Ahonen (2004)
		AY462338		Costa Rica, 2000, *Piippo s.n.* (H)
Austrolejeunea nudipes (Hook. f. & Taylor) Grolle	AY302445*		AY251171*	Ahonen (2004)
		AY462339		Australia, *Pócs & Streimann 99147/H* (CANB)
Cheilolejeunea imbricata (Nees) S. Hatt.	*AY125935*		*AY144475*	Ahonen et al. (2003)
Cololejeunea macounii (Spruce ex Underw.) A. Evans	*AY125942*		*AY144483*	Ahonen et al. (2003)
Lejeunea catanduana (Steph.) H. A. Mill., H. Whittier & B. Whittier	*AY125943*		*AY144484*	Ahonen et al. (2003)
		AY462363		China, *Koponen et al. 56008*
Lejeunea cavifolia (Ehrh.) Lindb.	*AY125945*		*AY144486*	Ahonen et al. (2003)
Lopholejeunea brunnea Horik.	*AY125930*		*AY144470*	Ahonen et al. (2003)
Mastigolejeunea auriculata (Wilson & Hook.) Schiffn.	*AY125933*		*AY144473*	Ahonen et al. (2003)
		AY462371		China, *Koponen et al. 51516*
Nephelolejeunea hamata (Hook. f. & Taylor) Grolle	AY305455*		AY251181*	Ahonen (2004)

Table 1. Continued.

Taxon	GenBank accession no. *rbc*L	*rps*4	*trn*L-F	Voucher
Nipponolejeunea pilifera (Steph.) S. Hatt.	*AY125937*		*AY144478*	Ahonen et al. (2003)
		AY462376		Japan, *Onhishi s.n.* (H)
Ptychanthus striatus Nees		AY462389		China, *Koponen et al. 57218*
			AY144471	Ahonen et al. (2003)
Trocholejeunea sandvicensis Mizut.	*AY125934*		*AY144474*	Ahonen et al. (2003)
		AY462401		China, *Koponen et al. 55768*
Order Radulales				
Radula complanata (L.) Dumort.	AY302461*		AY251188*	Ahonen (2004)
		AY462390		Finland, *Ahonen & Engblom 14*
Radula okamurana Steph.	AY302462*		AY251189*	Ahonen (2004)
		AY462391		Japan, *Ahonen 221*
Order Pleuroziales				
Pleurozia conchifolia (Hook. & Arn.) Austin	AY462324	AY462385	AY463582	Papua New Guinea, *Koponen 29264* (H)
Pleurozia gigantea (F. Weber) Lindb.	AY462325	AY462386	AY463583	Papua New Guinea, *De Sloover 42824* (H)
HORNWORTS				
Anthoceros formosae Steph.	*AB087449*	*AB087443*	*NC_004543*	Kugita et al. (2003)
Anthoceros punctatus L.	*APU87063*			Lewis et al. (1997)
		AJ250117		Capesius & Bloecher (unpublished)
MOSSES				
Andreaea rothii F. Weber & D. Mohr	*AF478200*	*AF478249*	*AF478295*	Magombo (2003)
Bryum argenteum Hedw.	*AY163024*			Pedersen & Hedenäs (2002)
		AY078318	*AY078291*	Cox & Hedderson (1999)
Buxbaumia viridis (DC.) Moug. & Nestl.	*AF478210*	*AF478253*	*AF478300*	Magombo (2003)
Dicranum scoparium Hedw.	*AF478226*	*AF478280*		Magombo (2003)
			AF234159	Newton et al. (2000)
Diphyscium foliosum (Hedw.) D. Mohr	*AF478220*	*AF478265*	*AF478303*	Magombo (2003)
Hedwigia ciliata (Hedw.) P. Beauv.	*AF231073*			Mishler et al. (unpublished)
		AF478289	*AF478336*	Magombo (2003)
Hypnum lindbergii Mitt.	*AB029390*			Tsubota et al. (1999)

Table 1. Continued.

Taxon	GenBank accession no. *rbc*L	*rps*4	*trn*L-F	Voucher
		AF143035	*AF161128*	Buck et al. (2000)
Mnium hornum Hedw.	*AF226820*			Wheeler et al. (unpublished)
		AF023796	*AF023767*	Cox & Hedderson (1999)
Polytrichum commune Hedw.	*U87087*			Lewis et al. (1997)
		AF208428		Hyvönen et al. (1998)
			AF231907	Newton et al. (2000)
Sphagnum cuspidatum Ehrh. ex Hoffm.	*AF478201*		*AF478292*	Magombo (2003)
		AF231893		Newton et al. (2000)
Sphagnum palustre L.	*AF231887*	*AF231892*	*AF231902*	Newton et al. (2000)
Takakia lepidozioides S. Hatt. & Inoue	*AF244565*			Parkinson et al. (1999)
		AF306950		Goffinet et al. (2001)
Tetraphis pellucida Hedw.	*AF478203*		*AF478297*	Magombo (2003)
		AF231896		Newton et al. (2000)
VASCULAR PLANTS				
Huperzia selago (L.) Bernh. ex Schrank & Mart.	*Y07934*			Wikström & Kenrick (1997)
			AJ224592	Wikström et al. (1999)
Lycopodiella inundata (L.) Holub	*Y07938*			Wikström & Kenrick (1997)
			AJ224605	Wikström et al. (1999)
Oryza sativa L.	*X15901*			Hiratsuka et al. (1989)
		NC_001320	*NC_001320*	Shimada & Sugiura (1991)
Pinus thunbergii Parl.	*NC_001631*	*NC_001631*	*NC_001631*	Wakasugi et al. (1994)
Psilotum nudum (L.) P. Beauv.	*U30835*			Hasebe et al. (1995)
		AF313588		Pryer et al. (2001)
			NC_003386	Wakasuki et al. (unpublished)
Pteridium aquilinum (L.) Kuhn	*U05939*			Wolf et al. (1994)
		AF197098		Speer (unpublished)
GREEN ALGAE				
Chlorella vulgaris Beij.	*AF499684*			Hayden & Waaland (2002)
		NC_001865	*NC_001865*	Wakasugi et al. (1997)
Coleochaete orbicularis Pringsh.	*L13477*			Manhart (1994)
			AF182368	Besendahl et al. (2000)
Nephroselmis olivacea F. Stein	*AF137379*	*AF137379*	*AF137379*	Turmel et al. (1999)

was calculated as the percentage of missing bases of the bases estimated to have been present in aligned matrix. The exact percentage of missing data is unknown, due to variability in the length of *rps*4 and especially *trn*L sequences. The percentage of indels is dependent on the alignment and, thus, changes according to the alignment method and parameter costs. Moreover, the natural length variation due to insertions and deletions was not scored as missing data.

DNA EXTRACTIONS

The specimens were ground in micro tubes that were kept in liquid nitrogen. DNA was extracted from the tissue using NucleoSpin Plant extraction kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instructions. The DNA was eluted in 60 μl of elution buffer. The DNA solution was diluted to 1/20–1/50 of the original concentration according to the DNA content, which was measured with Gene Quant Pro RNA/DNA Calculator (Amersham Pharmacia, Piscataway, New Jersey, U.S.A.). No other cleaning procedure was performed.

PCR AND SEQUENCING

Double stranded DNA templates were prepared using the polymerase chain reaction (PCR), employing 35 cycles of denaturation of the template DNA for 1 minute at 95°C, primer annealing for 1 min. at 49°C, primer extension for 1 min. at 72°C, preceded by an initial denaturation step for 10 min. at 95°C, and followed by a final extension step of 7 min. at 72°C. PCR was performed in 25 μl reaction volumes with the reaction mix optimized for liverworts (Ahonen et al., 2003) using the following primer combinations: *trn*L-*trn*F: *trn*C and *trn*F (Taberlet et al. 1991), *rbc*L: *rbc*LF and *rbc*LR (Ahonen et al., 2003), as well as 665F and 745R (La Farge et al., 2000), and *rps*4: trnas and *rps*5 (Cox & Hedderson, 1999). The resulting double-stranded PCR products were cleaned with GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia) and eluted in 20–50 μl of sterile water. Sequence reactions were prepared using the ABI PRISM Big Dye 1.0 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, U.S.A.) following the manufacturer's instructions. The same primers were used for sequencing. Sequence products were resolved on the MegaBACE automated sequencing machine (Amersham Biosystems).

Electropherograms were edited and forward and reverse sequences assembled for each DNA region using Sequencher version 4.1.2. (Gene Codes Corporation, Ann Arbor, Michigan, U.S.A.). The electropherograms obtained with the forward and reverse primers were compared with each other. To reject erroneous interpretations, the electropherograms from different species were also compared.

PHYLOGENETIC ANALYSIS

Direct optimization

Parsimony analysis was performed using direct optimization (Wheeler, 1996), implemented in the program POY version 3.0.6 (Wheeler et al., 2002). In the direct optimization procedure, the alignment of sequences and the search for the shortest tree are done simultaneously. The insertions and deletions are treated as processes, and homologies are dynamically determined and uniquely tailored for each topology (Wheeler, 2001). In order to find the most parsimonious alignment and tree, the program calculates the tree lengths of several million different character optimizations. The user can set the gap, transversion, and transition costs, and the length of the tree is the sum of all the substitution and insertion/deletion events that produce the tree in question. For more detailed explanation of the procedure see Wheeler (1996) and Schulmeister et al. (2002).

Direct optimization is computationally very demanding, because several million different alignments are produced and their costs estimated. Cutting sequences into shorter fragments can save dramatically on computation time and space when using optimization alignment (Wheeler et al., 2002). However, cutting the data into subfragments can affect the resulting tree lengths. In addition, choosing the cutting points introduces an extra degree of subjectivity to the analysis (Giribet, 2001). To avoid this, only the *trn*L-*trn*F data set was cut into subfragments. *Trn*L was cut for two reasons. First, many sequences from GenBank contained only the intron of *trn*L gene, whereas our own sequences also contained exon 2 of *trn*L as well as the partial *trn*F gene. This would have caused huge length differences between the sequences, which would be likely to cause ambiguity in the analysis (Belshaw & Quicke, 2002; Caterino & Vogler, 2002; Ahonen, 2004). To avoid this, the data sets were cut between the intron and exon 2 of the *trn*L. We assumed that treating the missing exon 2 and *trn*F parts as missing sequences in a separate data set would cause fewer problems in the analysis than sequences of vastly different length. A few bases of exon 1 were allowed to remain in the beginning of the intron data set. The second reason for cutting

*trn*L in subfragments was to ensure the biological plausibility of the homology statements in POY alignments. To ensure this, also exon 2 of *trn*L, the intergenic spacer between *trn*L and *trn*F, and the partial *trn*F were assigned to separate data sets. After preliminary runs we decided to leave out the intergenic spacer region (keeping the *trn*F data set) because of the considerable length variation that, as expected, seemed to cause spurious taxonomic groupings. We considered that the results would be more reliable without the effect of this region (Belshaw & Quicke, 2002; Caterino & Vogler, 2002; Ahonen, 2004).

In order to cut the sequences in subfragments, we first aligned the sequences with ClustalW version 1.8 (Thompson et al., 1994). The length variation in the beginning and end of the sequence was filled with Xs to avoid leading and trailing gaps, which would require a special cost set to them (Schulmeister et al., 2002), and having to assess the effects of changes in this parameter value as well. The beginnings and ends of the *trn*L-F fragment are in very conservative areas, where the biologically correct alignment is easy to assess even by eye, so the missing bases could be filled reliably with Xs. POY reads the X as an unknown base or a gap. The internal gaps were removed from the subfragments before the POY analysis.

That the *rbc*L and *rps*4 data sets were left uncut led to increased computational requirements, and the analyses could only be run in a parallel supercomputer of the CSC—Scientific Computing Ltd. (CSC = Finnish IT Center for Science) using 16 processors of 1.1 GHz each. Each analysis took a minimum of three days. All the data sets were run simultaneously. We assessed the effects of four different parameter combinations (gap : transition : transversion cost), 1:1:1, 2:1:1, 2:2:1, and 4:1:1.

Because POY optimizes the characters to support certain topology (the shortest tree), in some cases it is parsimonious to insert single or more gaps even in coding regions (Ahonen, 2004). Therefore the *rbc*L data set was submitted as prealigned both to ensure the biologically correct alignment and to save computation time. The POY command line applied is given below in bold.

poy -molecularmatrix Filename -norandomizeoutgroup -holdmaxtrees 20 -maxtrees 5 -fitchtrees -multibuild 10 -random 5 -seed -1 -slop 3 -checkslop 30 -tbr -spr -sprmaxtrees 5 -drifttbr -numdrifttbr 5 -ratchetspr 5 -fuselimit 50 -fusingrounds 1 -impliedalignment -indices -repintermediate -poytreefile Filename -poybintreefile Inputfiles >Outfile.out

More information can be found in the POY manual, available from the American Museum of Natural History website: ⟨//ftp.amnh.org/pub/people/wheeler/poy/poy.pdf⟩.

We explored the effects of the four different parameter value combinations (1:1:1, 2:1:1, 2:2:1, and 4:1:1), because the cost parameters may have a great effect on results (e.g., Giribet, 2001; Schulmeister et al., 2002; Ahonen, 2004). It is impossible to know a priori which parameters give the correct topology or to know which of the different topologies obtained reflects the true phylogeny. Wheeler (1995) proposed sensitivity analysis as one solution to the problem. In sensitivity analysis the alignments and trees produced with different gap costs are evaluated by their congruence either with accepted monophyletic groups based on morphology (= taxonomic congruence sensu Wheeler, 1995, *non* Miyamoto & Fitch, 1995) or by the congruence of different data subsets used in the same analysis (Wheeler, 1995, 2001). The latter is analogous to looking for greatest congruence among characters.

We assessed the congruence between data sets by ILD (incongruence length difference) (Mickevich & Farris, 1981; Farris et al., 1995). To get the necessary values to calculate ILD values, every genomic region (*rbc*L, *trn*L-F, and *rps*4) was analyzed separately with every parameter combination.

NONA ANALYSIS

To test the results given by POY, we also analyzed the most parsimonious implied alignment of the POY tree obtained with parameter values 1:1:1 with the parsimony program Nona. Using the POY alignments in other programs that require aligned sequences has often been done (e.g., Wahlberg & Zimmermann, 2000; Belshaw & Quicke, 2002). Wheeler (2003a) recommends this to check that POY has found the shortest tree. Alignments of different data subfractions were combined in Winclada (Nixon, 2002) and submitted to Nona version 1.8 (A. P. Goloboff). The Nona command line was **hold 100; hold/20; mult*30; duplicate*; jump 5; sswap 4; length*; suboptimal 1; unique; pack.**

Gaps that were the consequence of the natural insertions and deletions were treated as a fifth character state in the analysis. The two different types of gaps were coded differently: the deletions as state 5 and the missing data as question marks.

Table 2. Tree attributes with different gap : transversion : transition costs in POY.

Gap : transversion : transition costs used in the analysis	Tree length	Tree no.	ILD	CI	RI	Informative characters (%)
1 : 1 : 1	14692	1	0.04	0.28	0.64	2044/3693 (55)
2 : 2 : 1	21398	1	0.05	0.26	0.64	1874/3439 (54.5)
2 : 1 : 1	16981	1	0.06	0.22	0.61	1717/3020 (57)
4 : 2 : 1	25332	1	0.07	0.21	0.61	1738/2999 (58)

Contiguous gaps were treated as if they were composed of individual equally weighted characters.

BREMER SUPPORT VALUES

Because the topology of the Nona tree was exactly the same as the 1 : 1 : 1 POY tree (which was also the best tree according to the ILD values, see Results below), we calculated the Bremer support values (Bremer, 1988) with Nona. The advantage of calculating the Bremer support values with Nona rather than with POY is that in Nona it is much easier to use several rounds of branch swapping, as well as to use thousands of trees as the starting trees for the Bremer search. According to our experience, both of these factors affect the correctness of the Bremer support values.

We calculated the Bremer support values in the following way: first, we searched gradually longer trees, in order not to fill the tree buffer only with longer trees, which are of course easiest to find. When there were 32,000 trees in memory (Nona version 1.8 can only count Bremer support values for 32,670 trees, according to its own error message), the command **bsupport** was given without an argument. Thus, the program counted the Bremer support values using the trees in memory.

RESULTS

MOLECULAR MARKERS

The length of the longest and the shortest unaligned sequences are given below to give an idea of the length variation in each gene.

1. *rbc*L. We used 1038 base pairs of the gene for the large subunit of the ribulose bisphosphate carboxylase (*rbc*L) gene. The amount of the missing data in *rbc*L data set was 7%. There is no length variation in *rbc*L sequences.

2. *trn*L-*trn*F. This chloroplast fragment contains a partial sequence of the leucine transfer $RNA_{(UAA)}$ (which is composed of 2 exons and an intron), an intergenic spacer, and a partial sequence of phenylalanine $tRNA_{(GAA)}$. We did not use the intergenic spacer in the analyses. The amount of missing data was about 10%. The longest complete sequence in the intron data set was that of *Lycopodiella inundata* with 574 base pairs, and the shortest that of *Sphaerocarpos texanus* with 226 base pairs. In the *trn*L exon 2 data set the longest complete sequences were from *Sphagnum* L. (2 species; 53 bp) with the shortest those of *Andreaea rothii* and *Mnium hornum* (both at 49 bp). In the *trn*F data set the longest sequence was that of *Makinoa* sp. ined. 1 (39 bp) and shortest that of *Schistochila laminigera* (35 bp).

3. *rps*4. This gene codes for the chloroplast ribosomal protein S4. The *rps*4 primers (Cox & Hedderson, 1999) isolate a region that contains from 5′ end incomplete *rps*4 gene as well as part of the intergenic spacer between *rps*4 and the gene for serine transfer RNA. We included only the *rps*4 gene in the analyses. There are some variable regions in *rps*4, the longest sequence being that of *Chlorella vulgaris* with 591 basepairs and the shortest that of *Pinus thunbergii* with 564 basepairs.

TAXONOMY

With each parameter combination applied, the POY analyses resulted in one most parsimonious tree only. According to the ILD values, the best POY tree was the one obtained with the parameters (gap : transversion : transition cost) 1 : 1 : 1, with value 0.04 and CI = 0.28, RI = 0.64 (Table 2, Fig. 1). The second best tree was the 2 : 2 : 1 tree, while the 2 : 1 : 1 and 4 : 2 : 1 trees (trees not shown) were clearly worse (Table 2). In all the trees the liverworts were resolved as a monophyletic group, with *Blasia pusilla* sister to all other liverworts (Table 3). The position of the two *Haplomitrium* species varied among the trees; sometimes they were among the simple thalloid liverworts, and sometimes the genus appeared among the vascular plants. Mosses and vascular plants were paraphyletic in the trees obtained with all the parameter combinations. How-

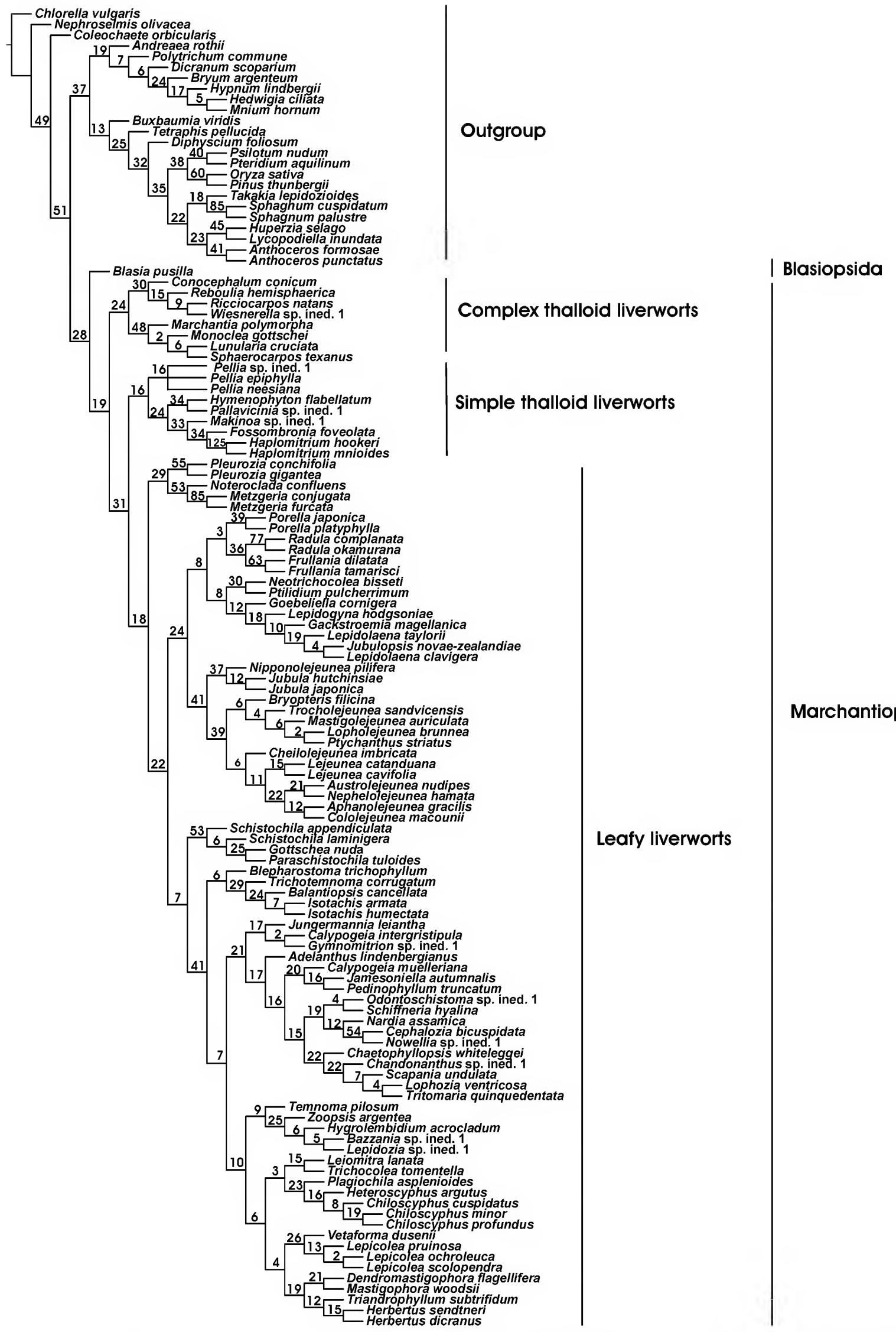

Figure 1. The most congruent tree, obtained with the optimization parameters 1:1:1 using POY. The Bremer support values are shown above each node, CI = 0.28, RI = 0.64.

Table 3. Phylogenetic status of groups among liverworts with different gap : transversion : transition costs in POY.

Gap : transversion : transition costs used in the analysis	Liverwort clade	Complex thalloid liverworts	Simple thalloid liverworts (excluding *Metzgeria* and *Noteroclada*)	Acrogynous liverworts	Liverworts with true leaves	Jungermannioid leafy liverworts clade	Lepicoleineae R. M. Schust.
1 : 1 : 1	Monophyletic	Monophyletic	Monophyletic	Monophyletic	Paraphyletic	Monophyletic	Monophyletic
2 : 2 : 1	Monophyletic (except *Haplomitrium*)	Monophyletic	Paraphyletic	Polyphyletic	Polyphyletic	Monophyletic	Monophyletic
2 : 1 : 1	Monophyletic (except *Haplomitrium*)	Polyphyletic	Polyphyletic	Polyphyletic	Paraphyletic	Paraphyletic	Monophyletic
4 : 2 : 1	Monophyletic (except *Haplomitrium*)	Polyphyletic	Polyphyletic	Polyphyletic	Paraphyletic	Paraphyletic	Monophyletic

Herbertineae R. M. Schust.	Lepidoziineae R. M. Schust.	Cephaloziineae R. M. Schust.	Jungermanniineae R. M. Schust.	Geocalycineae R. M. Schust.	Perssoniellineae R. M. Schust.	Balantiopsidineae R. M. Schust.	Pleuroziineae R. M. Schust.
Polyphyletic	Polyphyletic	Polyphyletic	Polyphyletic	Polyphyletic	Monophyletic (with *Gottschea nuda*)	Monophyletic	Monophyletic
Polyphyletic	Polyphyletic	Polyphyletic	Polyphyletic	Polyphyletic	Monophyletic (with *Gottschea nuda*)	Monophyletic	Monophyletic
Polyphyletic	Polyphyletic	Polyphyletic	Polyphyletic	Polyphyletic	Polyphyletic	Monophyletic	Monophyletic

Radulineae R. M. Schust.	Ptilidiineae R. M. Schust.	Lepidolaenineae R. M. Schust.	Porellineae R. M. Schust.	Lepicoleales Stotler & Stotl.-Crand.	Jungermanniales H. Klinggr. emend. Stotler & Stotl.-Crand. monophyletic	Isophylly found in all the major leafy liverwort lineages	Anisophylly found in all the major leafy liverwort lineages	Position of *Haplomitrium*
Monophyletic	Polyphyletic	Paraphyletic	Paraphyletic	Polyphyletic	Paraphyletic	No	Yes	Among simple thalloids
Monophyletic	Polyphyletic	Polyphyletic	Paraphyletic	Polyphyletic	Paraphyletic	No	Yes	In the outgroup
Monophyletic	Polyphyletic	Paraphyletic	Paraphyletic	Polyphyletic	Paraphyletic	No	Yes	In the outgroup
Monophyletic	Polyphyletic	Polyphyletic	Paraphyletic	Polyphyletic	Paraphyletic	No	Yes	In the outgroup

ever, our sampling among these outgroup clades was scattered and incomplete, so we prefer not to draw any conclusions of their taxonomy. In the following description of the results, the current taxonomic status of the species is referred to the systematic concept of Crandall-Stotler and Stotler (2000).

The complex thalloid liverworts form a monophyletic clade, which is divided into two sister clades, one with *Marchantia polymorpha* and *Lunularia cruciata* (subclass Marchantiidae Engl.), *Monoclea gottschei,* and *Sphaerocarpos texanus* (subclass Sphaerocarpidae Stotler & Stotl.- Crand.). The second clade contains *Conocephalum conicum, Reboulia hemisphaerica,* and *Wiesnerella* sp. ined. 1 and *Ricciocarpos natans.*

The simple thalloid liverworts form a monophyletic clade between the complex thalloid and leafy liverworts. They include the *Fossombronia foveolata* and *Pellia* Raddi (3 species) (subclass Metzgeriidae Barthol.-Began), *Hymenophyton flabellatum, Pallavicinia* Gray (1 species), *Makinoa* Miyake (1 species), and *Haplomitrium* (2 species). *Noteroclada confluens, Metzgeria conjugata,* and *M. furcata* group with the leafy liverwort *Pleurozia gigantea* and *P. conchifolia* (subclass Jungermanniidae Engl. emend. Stotler & Stotl.-Crand.), and they form a sister clade to all the other leafy liverworts. The positions of *Haplomitrium, Noteroclada confluens,* and *Metzgeria* are noteworthy here because the position of *Haplomitrium* has been controversial (e.g., Schuster, 1979; Crandall-Stotler & Stotler, 2000), and *Noteroclada* Taylor ex Hook. & Wilson and *Metzgeria* have been considered as simple thalloid liverworts (e.g., Schuster, 1979; Crandall-Stotler & Stotler, 2000).

Above the *Pleurozia–Noteroclada–Metzgeria* clade, the rest of the leafy liverworts are divided into two sister clades: one of them containing the *Frullania* Raddi (2 species), *Goebeliella cornigera, Jubula* Dumort. (2 species), *Nipponolejeunea pilifera, Porella* L. (2 species), the Lejeuneaceae Casarea-Gil. (12 species), *Ptilidium pulcherrimum* (Ptilidiineae R. M. Schust.), *Radula* Dumort. (2 species), *Gackstroemia magellanica, Jubulopsis novae-zealandiae, Lepidogyna hodgsoniae, Lepidolaena* Dumort., and *Neotrichocolea bisseti* (Lepidolaenineae R. M. Schust.). This group is further divided into two sister clades, one of them consisting only of Lejeuneaceae, *Jubula,* and *Nipponolejeunea* S. Hatt., whereas the other clade contains other Porellales, all the Lepidolaenineae, *Radula,* and *Ptilidium* Nees. *Nipponolejeunea pilifera* groups with genus *Jubula* rather than the Lejeuneaceae. *Frullania* groups with *Radula. Ptilidium* and *Neotrichocolea* form a sister group to *Goebeliella* Steph. and Lepidoleanineae.

Another main clade contains all the remaining taxa included. *Schistochila* Dumort. (2 species), *Paraschistochila tuloides,* and *Gottschea nuda* (Perssoniellineae R. M. Schust.) are sister to the rest of the leafy liverworts. *Blepharostoma trichophyllum* and *Trichotemnoma corrugatum,* as well as *Balantiopsis cancellata* and *Isotachis* Mitt. (2 species) (Balantiopsidineae R. M. Schust.), form a sister group to the remaining liverworts, which again are divided in two clades. One of them contains mostly species from the order Jungermanniales: *Cephalozia bicuspidata, Nowellia* sp. ined. 1, *Odontochistoma* sp. ined. 1, *Adelanthus lindenbergianus* and *Schiffneria hyalina* (Cephaloziineae R. M. Schust.), *Jungermannia leiantha, Jamesoniella autumnalis, Nardia assamica, Chandonanthus* sp. ined. 1, *Lophozia ventricosa, Tritomaria quinquedentata, Gymnomitrion* sp. ined. 1, *Scapania undulata* (Jungermanniineae R. M. Schust. ex Stotler & Stotl.-Crand.), *Calypogeia integristipula* and *C. muelleriana,* (Lepidoziineae R. M. Schust.), and *Pedinophyllum truncatum* (Lophocoleineae Schljakov.). Only *Chaetophyllopsis whiteleggei* (Ptilidiineae R. M. Schust.) represents the order Lepicoleales here.

The other sister clade contains mostly species from those suborders and orders that have been traditionally considered basal either in Jungermanniales or Lepicoleales (Schuster, 1979; Crandall-Stotler & Stotler, 2000): *Dendromastigophora flagellifera, Mastigophora woodsii* (Ptilidiineae), *Plagiochila asplenioides, Heteroscyphus argutus, Chiloscyphus* Corda (3 species) (Lophocoleineae Schljakov) , *Vetaforma dusenii, Lepicolea* Dumort. (3 species) (Lepicoleineae R. M. Schust.), *Leiomitra lanata, Trichocolea tomentella* (Lepidolaenineae), *Herbertus sendtneri* and *H. dicranus, Temnoma pilosum* and *Triandrophyllum subtrifidum* (Herbertineae R. M. Schust.), *Zoopsis argentea, Hygrolembidium acrocladum, Bazzania* sp. ined. 1, and *Lepidozia* sp. ined. 1 (Lepidoziineae R. M. Schust).

The 2:2:1 POY tree (not shown) is the second best, according to the ILD values (0.05), among the POY trees obtained with different parameter costs (Table 2). For the 2:2:1 POY tree the CI is 0.26 and RI is 0.64. There are some differences inside the complex thalloid clade between this tree and the 1:1:1 tree, but the complex thalloid clade is still monophyletic and divided into the same two sister clades. However, the POY trees differ with regard to the simple thalloid liverworts and leafy liverworts. In the 2:2:1 POY tree the *Pleurozia–Noteroclada–Metzgeria* clade is now between the

complex thalloid liverworts and the simple thalloid liverworts, which are paraphyletic. The *Pellia* species form a clade of their own, as do *Fossombronia* Raddi, *Makinoa*, *Hymenophyton* Dumort., and *Pallavicinia*. Genus *Haplomitrium* is outside the liverwort clade, between the *Pteridium–Psilotum* and the *Takakia–Sphagnum* clades.

The leafy liverworts divide into two sister clades, but there are some differences inside these clades in 1:1:1 and 2:2:1 (tree not shown) POY trees. In the 2:2:1 POY tree the *Ptilidium–Neotrichocolea* branch is the most basal in the Porellales–Radulales–Lepidolaenineae clade (Table 1, see Discussion). The *Frullania–Radula* clade comes next, and the rest is divided to two clades: the first containing again *Jubula*, *Nipponolejeunea*, and the Lejeuneaceae, and the other *Porella*, *Goebeliella*, and the Lepidolaenineae. In the other leafy liverwort clade the *Schistochila–Paraschistochila–Gottschea* clade is still the most basal, but now *Blepharostoma trichophyllum* alone is sister to the rest of the species. The remaining taxa are still divided into two sister clades, but the internal taxonomy of both of them differs markedly from the 1:1:1 tree (Fig. 1). *Trichotemnoma* R. M. Schust., *Balantiopsis* Mitt., and *Isotachis* Mitt. move to one of the clades and form a sister clade to *Jungermannia leiantha*, *Calypogeia integristipula*, and *Gymnomitrion* Corda.

The 2:1:1 tree had ILD = 0.06, CI was 0.22, and RI 0.61 (Table 2). In this tree (not shown) the complex thalloid liverwort clade becomes paraphyletic, when *Sphaerocarpos*, *Lunularia* Adans., *Marchantia* L., and *Monoclea* Hook. jump to an apical position among the leafy liverworts. The simple thalloid liverworts are basal in the two sister clades of the leafy liverworts; *Hymenophyton*, *Pallavicinia*, *Fossombronia*, and *Makinoa* are basal in the Porellales–Radulales–Lepidolaenineae clade, and *Pellia* and *Noteroclada* Taylor ex Hook. & Wilson together with *Pleurozia* are basal in the other clade. *Metzgeria*, however, is among the leafy liverworts together with the complex thalloid liverworts. *Haplomitrium* is outside of the liverwort clade, now between *Takakia–Sphagnum* clade and *Huperzia* Bernh. There are further differences in the tree compared to the 1:1:1 tree inside the leafy liverwort clades. Among other things, *Frullania* and *Radula* now group with the *Jubula* and Lejeuneaceae, and the *Blepharostoma–Trichotemnoma–Balantiopsis–Isotachis* clade moves to the clade containing most of the Lepicoleales.

The 4:2:1 tree had ILD = 0.07, the CI was 0.21 and RI 0.61 (Table 2). In this tree (not shown) the complex thalloid liverworts are paraphyletic as in the 2:1:1 tree (not shown). The simple thalloid liverworts remain paraphyletic, but now *Pallavicinia* and *Pellia* return to the stem position below the leafy liverworts. *Pleurozia* and *Noteroclada* are sister to the rest of the leafy liverworts; *Hymenophyton*, *Fossombronia*, and *Makinoa* are sister to the Porellales–Radulales–Lepidolanineae clade. *Metzgeria* groups together with some complex thalloid species in an apical position in leafy liverworts. *Pleurozia* groups with *Noteroclada*, and *Haplomitrium* is between the *Takakia–Sphagnum* clade and *Huperzia*. Except for the simple thalloid liverworts, the *Neotrichocolea–Ptilidium* clade is now sister to the rest of the Porellales–Lepidolaenaceae–*Ptilidium* clade. *Frullania–Radula* branch groups again with the *Jubula* and Lejeuneaceae instead of the *Porella–Goebeliella–Jubulopsis*–Lepidolaenineae clade. The *Schistochila–Gottschea* clade is sister to clade containing the rest of the liverworts, while *Paraschistochila* R. M. Schust. is sister to *Metzgeria* and the complex thalloids.

The tree that Nona produced from the implied alignment of the 1:1:1 POY tree was exactly the same as the POY tree with respect to the topology. However, Nona gave a slightly shorter tree length, (14,685 instead of 14,692) and slightly different CI and RI values, 0.26 and 0.56, respectively. The differences between the POY and Nona tree lengths could be due to the heuristics of different cladogram search procedures (Wheeler, 2003a) or the fact that the direct optimization algorithm is not exact and the direct optimization method provides an upper bound on the true minimum cost (Wheeler, 2003b). Even the CI and RI values in POY are estimates and may, therefore, differ from the CI and RI values given by Nona (POY manual: ⟨//ftp.amnh.org/pub/people/wheeler/poy/poy.pdf.⟩).

Discussion

On methodology

We analyzed our data with the direct optimization (DO) method (Wheeler, 1996), which we have found the least subjective way to handle sequences that are difficult to align (especially *trn*L-F data in our case) and by far the most efficient way to find the most parsimonious alignment and the tree. This program is able to try millions of different alignments in contrast to existing multiple alignment programs that produce only one static alignment. Often regions difficult to align unambiguously with these multiple alignment programs, or to align manually, are left out from the analysis; this is even recommended (Mindell & Honeycutt, 1990; Swofford et al., 1996; Naylor & Brown, 1997). We con-

sider this a waste of data, and consider that POY has the ability to use the signal hidden in even highly variable regions, aligning them, if possible, according to the strongest signal in the data. However, at the same time, POY may be sensitive to non-homologous insertions and deletions especially with higher gap costs (Belshaw & Quicke, 2002; Caterino & Vogler, 2002; Ahonen 2004). This is obviously a consequence of POY treating gaps as fifth character states. To avoid this, we left out the two gene regions with high length differences that might have caused problems in the analyses, the intergenic spacers between *trn*L and *trn*F and between *rps*4 and *trn*S.

Both the strength and the weakness of POY is that it optimizes the characters across all data, forcing the alignment to support a certain topology. The strength of this method is that POY is thus able to find hidden support in such data sets, where the true signal may have been obscured by a random signal (Cognato & Vogler, 2001). Its weakness is that the optimization of single characters is not independent of the optimization of the other characters. However, we find the advantages of using POY outweigh this disadvantage.

We tried the effect of several different parameter costs on the results. Once again, our results show that the parameters used have a great effect on tree topology (see also Giribet, 2001; Schulmeister et al., 2002). Judged by the ILD values in the present study, the data sets became less congruent when the gap cost became higher. Also, there was less taxonomic congruence with earlier hypotheses of the taxonomic groupings. This could be due just to the tendency of POY to become more sensitive to the insertions and deletions in the data, when the gap cost is increased (Belshaw & Quicke, 2002; Caterino & Vogler, 2002; Ahonen, 2004). We chose the tree obtained with the parameters 1:1:1 as the best result. We base this choice primarily on the best ILD value (Table 2) that reflects the character congruence of Schulmeister et al. (2002) and Wheeler (1995, 2001), although there is some evidence that the ILD values are not a reliable method to find the accurate phylogeny (Yoder et al., 2001; Dowton & Austin, 2002; see also Grant & Kluge, 2003, for philosophical discussion). However, we consider that 1:1:1 is the most parsimonious choice of parameters, as it contains no ad hoc decisions of the frequency of different changes in the DNA (see Allard & Carpenter, 1996; Allard et al., 1999) and because it maximizes congruence over *all* data by minimizing the *total* number of hypothesized transformations (Grant & Kluge, 2003). Nona returned the same tree as POY when the implied alignment of the 1:1:1 tree was used as static alignment, although with slightly different length (14,685 instead of 14,692, see Results).

In addition, the 1:1:1 tree is best because it has the highest congruence with accepted taxonomic groupings based on morphological data (taxonomic congruence of Wheeler, 1995). The concept of taxonomic congruence is, however, not philosophically unproblematic—Wheeler himself stated (Giribet & Wheeler, 1999: 135): "Taxonomic congruence as used by Wheeler (1995) is probably not a good criterion to choose among competing hypotheses because the groups have to be defined arbitrarily (perhaps based on current taxonomy)." There is a clear danger of circularity when the "right" result is chosen by its congruence with earlier results, and then the same characters that defined the earlier result are again "found" to support the new result.

In our results, the higher gap costs raised the ILD values, indicating higher incongruence between the data sets. This is opposite to the effect of gap costs on ILD values reported by Dowton and Austin (2002). Also, the lesser degree of congruence of the cladograms obtained with higher gap costs with the most widely accepted taxonomic groupings seem to indicate that in the present study the ILD values truly reflect the degree of accuracy in taxonomic inference. The higher (= worse) ILD value a tree has, the more peculiar the groupings, in that they do not match any morphological synapomorphies. One example of this is the position of the complex thalloid liverworts as the most apical branch among the leafy liverworts in the 2:1:1 and 4:1:1 trees. Surprisingly, the 2:2:1 tree is the second best tree, better than the 2:1:1 tree, in terms of both character and taxonomic congruence, although weighting gaps and transversions equally is not usual in phylogenetic studies. It is more often used when a larger parameter space is explored for methodological purposes (e.g., Wheeler, 1995; Giribet, 2001; Schulmeister et al., 2002). However, this is in accordance with Ahonen (2004). In most studies only the effect of changes in the gap cost is assessed, while the transversion:transition cost is kept equal.

CI and RI values, which measure the amount of homoplasy in the data, indicate that the homoplasy increases when the gap costs are raised. The 1:1:1 tree has the highest (= best) CI and RI values, i.e., homoplasy in the data is lowest in this tree.

There is not much difference in the percentage of informative characters between the alignments obtained with the different parameter combinations (Table 2); however, the percentage of informative

characters increases with gap cost. This is due to a greater percentage of autapomorphies in alignments obtained with low gap costs.

SYSTEMATIC POSITION AND EVOLUTIONARY TRANSFORMATION OF LIVERWORTS

Our study shows that the liverworts, as a whole, are a monophyletic group and are sister to the rest of the land plant lineages. Their synapomorphies include the internalized sporophyte maturity (i.e., the sporophyte develops entirely within the confines of gametophyte tissue), the presence of elaters in the capsule, and the presence of lunularic acid (Mishler & Churchill, 1984). The occurrence of the oil bodies in about 90% of liverwort species (Crandall-Stotler & Stotler, 2000) may not be considered as a synapomorphy, since there are no oil bodies present in *Blasia pusilla,* the taxon that is sister to the rest of the liverworts. Our results suggest that the liverworts diverged first from the land plant ancestors, and this hypothesis is in agreement with Mishler and Churchill (1984, 1985), Kenrick and Crane (1997b) based on morphological data, Edwards et al. (1995) on fossil evidence, as well as Qiu et al. (1998) using mitochondrial genome evidence. As stated by Qiu et al. (1998), liverworts could serve as valuable models to study such important adaptation to life on land as the embryo, archegonium, and antheridium, as they are the first plants to possess these features.

Within the liverworts, the clades from *Blasia,* to complex thalloid, simple thalloid, and to leafy liverworts contribute our understanding of evolution and classification of these organisms. The strong variation in gametophyte architectures may be largely linked to the modification of the apical cell (Schuster, 1984a). Patterns in apical cell shape overlays with our phylogenetic hypotheses. In *Blasia* and in the marchantioid clade from *Conocephalum conicum* to *Sphaerocarpos texanus,* the apical cell is cuneate-shaped, with four cutting faces (Renzaglia, 1982). In the rest of the simple thalloid taxa, the apical cell can be cuneate, with four cutting faces (*Pellia* and *Makinoa*), lenticular, with two cutting faces (*Fossombronia, Hymenophyton, Metzgeria,* and *Pallavicinia*), tetrahedral, with three cutting faces (*Haplomitrium* and *Noteroclada*), or rarely hemidiscoid (*Pellia*), with three cutting faces (Renzaglia, 1982). In the leafy liverworts, the apical cell is tetrahedral, with three cutting faces, except in *Pleurozia,* which is lenticular (Crandall-Stotler, 1976). Based on our phylogenetic hypothesis, the cuneate apical cell likely represents the ancestral type of apical cell geometry in liverworts. This does not support Schuster's theory (1984a) that the apical cell evolved from the tetrahedral apical cell with three equal segments cut off, as in *Haplomitrium.*

Transformation from the cuneate to other types of the apical cell reflects also the shift in growth form from thalloid, dorsiventral to leafy, radial. Thus, the thalloid growth form, probably as in *Blasia,* has given rise to the leafy forms. Within the leafy liverworts, bilateral symmetry is the ancestral state and radial symmetry the derived state. The ancestral liverwort is likely to have been a simple thalloid gametophyte, with growth via a cuneate apical cell, and bearing a sporophyte with massive foot and seta, probably as those of *Blasia.* Therefore, Schuster's (1966, 1979, 1981, 1984a, 2000, 2002) long-standing opinion that the earliest liverworts had predominantly erect, radial gametophytes, formed from a tetrahedral apical cell cutting off equal segments (with dorsiventrality, anisophylly, and the development of thalloid gametophyte being later specializations) is not corroborated by our analysis. The growth form, whether thalloid and dorsiventral or leafy and radial, has been shown to be controlled by organographic potentials, which reside in meristematic, apical derivatives (Crandall-Stotler, 1981). Even though leaf development in the Jungermanniales and the simple thalloid Metzgeriales has been shown to be different (Crandall-Stotler, 1981; Renzaglia, 1982; Schuster, 1984a), two leaf initials were involved in the former and one central wedge cell of a three-celled lateral derivative are involved in the latter. In our results, *Noteroclada* and *Metzgeria* of the leafy Metzgeriales did not cluster together with other simple thalloid taxa, but with *Pleurozia* of the leafy Jungermanniales, and they form an independent clade. Morphologically, however, this close relationship can only be detected in the acrogynous archegonium position (see discussion in the section of simple thalloid liverworts) that occurs in all three of the above genera, and in that the lenticular apical cell occurs in both *Pleurozia* and *Metzgeria.* As stated by Renzaglia (1982), the development of lobe and lobule tissue from the three outer cells of a five-celled lateral merophyte occurs in both *Treubia* of the leafy Metzgeriales and *Pleurozia* of the Jungermanniales. Our result implies that the strict division of the leafy and thalloid taxa according to the number of merophyte cells should be re-evaluated.

ON *BLASIA PUSILLA*

In the present study, *Blasia pusilla* is resolved as sister to the rest of the liverwort species. One of

the earliest liverwort fossils, the Carboniferous *Blasiites* R. M. Schust., has a similar anatomy as extant *Blasia* (Schuster, 1966). Traditionally, the monotype *Blasia pusilla* (and also the genus *Cavicularia* Steph.) is assigned to the family Blasiaceae Klingg. in the Metzgeriales (see Renzaglia, 1982; Schuster, 1992). This arrangement is also supported by the 26S rRNA data (Wheeler, 2000). However, morphologically *Blasia* differs from all other simple thalloid members in having two rows of well-developed, many-celled ventral scales, and ventral auricles filled with *Nostoc* colonies. Various recent studies have questioned the systematic position of the genus. Duckett et al. (1982) emphasized that the microanatomy of the spermatozoid of *Blasia* is unique in Metzgeriales studied to date, and is closer to that of the Marchantiidae than the Jungermanniidae. In the cladistic analyses that include characters related to male gametogenesis (Garbary et al., 1993) and use five chloroplast genes (Forrest & Crandall-Stotler, 2004), *Blasia* is positioned together with the Marchantiopsida. Pass and Renzaglia (1995) placed the Blasiaceae in the Marchantiopsida because of the similarities of their shared blepharoplast structure in conjunction with other morphological data. Stech and Frey (2001) proposed to separate *Blasia* in its own class Blasiopsida, on the basis of *trn*L intron data, as an intermediate group between Marchantiopsida and Jungermanniopsida. The position of *Blasia* in our result is different from all these previous studies; nonetheless, it shows clearly that *Blasia* is very isolated from the rest of the liverworts. It deserves a more independent, taxonomic status. Therefore, our evidence supports *Blasia* in its own class Blasiopsida Stech & W. Frey (Stech & Frey, 2001), and suggests that the class be placed at the beginning of any classification scheme for the liverworts.

ON COMPLEX THALLOID LIVERWORTS

The complex thalloid liverworts, represented by the *Conocephalum–Sphaerocarpos* clade, are monophyletic. The monophyly of this group is also supported by other molecular studies (Lewis et al., 1997; Wheeler, 2000; Stech & Frey, 2001; Boisselier-Dubayle et al., 2002). The complex thalloid liverworts form a sister group to the simple thalloid and leafy liverworts. In the 1950s, the marchantioid complex of thalloid species were recognized as one of the two major groups of the liverworts based on morphological and ecological features (Schuster, 1953, 1958). Schuster has discussed at length the profound differences between the complex thalloid and the simple thalloid and leafy liverworts. For example, the six rows of archegonial neck cells, the unistratose capsule wall, and the dimorphic leaf cells are all unique to the complex thalloid liverworts. Schuster regarded the differentiation between the two groups as an initial dichotomy in liverwort evolution, and asserted that the evolution of the complex thalloid liverworts occurred considerably later than the split between simple thalloid and leafy liverworts (Schuster, 1981, 1984a). Our study partially confirms Schuster's account of evolutionary transformation in liverworts, i.e., that complex thalloid and simple thalloid and leafy liverworts represent clear separate lineages. However, in our study we find that only one initial group of liverworts evolved in early diversification, which is *Blasia pusilla,* the first lineage to diverge from the liverwort ancestor. The complex thalloid liverworts share with *Blasia* the ventral scales on gametophytes and also other characters (see above). Our study indicates that the complex and simple thalloid and leafy liverworts share a common, probably simple thalloid ancestor. The assumption by Schuster that the evolution of the complex thalloid liverworts was considerably later than that of the simple thalloid/leafy liverworts is not supported, considering their sister relationships.

Although not all families of the complex thalloid liverworts were sampled for the study, species from each major subgroup sensu Crandall-Stotler and Stotler (2000), the Sphaerocarpales, the Monocleales, and the Marchantiales, were included. Our results show that Marchantiales are paraphyletic, and Sphaerocarpales and Monocleales are clustered within Marchantiales. This result confirms that of the study based on morphological characters combined with sequence data of the nuclear 26S rDNA gene (Boisselier-Dubayle et al., 2002).

ON SIMPLE THALLOID LIVERWORTS

Morphologically, the simple thalloid liverworts are a strikingly diverse group, as can be seen in the gametophytes with leafy or thalloid growth, five types of apical cell geometry, and several types of capsule dehiscence. The group has been mostly regarded as paraphyletic in various phylogenetic studies (Lewis et al., 1997; Crandall-Stotler & Stotler, 2000; Stech et al., 2000; Stech & Frey, 2001; Forrest & Crandall-Stotler, 2004). We present a different viewpoint on the phylogeny of the simple thalloid liverworts, and propose that most of the simple thalloid liverworts are a monophyletic group.

Most species of the simple thalloid liverworts sampled cluster together as a monophyletic group,

except *Noteroclada* and *Metzgeria,* which are grouped together with the leafy genus *Pleurozia* as an independent clade. Although the simple thalloid Metzgeriales and the leafy Jungermanniales have been sharply divided by the character gametangial position (acrogynous or anacrogynous), some groups of Metzgeriales seem not to be truly anacrogynous. In the Metzgeriaceae H. Klinggr., Aneuraceae H. Klinggr., Hymenophytaceae R. M. Schust., Vandiemeniaceae Hewson, and Mizutaniaceae Furuki & Z. Iwats., the archegonia are restricted to highly abbreviated, lateral or ventral branches, which fail to innovate apically after archegonium formation (Schuster, 1964a, 1984a). In *Podomitrium* Mitt. of Pallaviciniaceae Mig., the archegonia are terminal. Thus, these groups are actually acrogynous, in common with the Jungermanniales. In *Noteroclada,* there are two types of archegonia: scattered archegonia along the midrib that are not functional; and terminal archegonia, which are very near the apical cell. In the latter case, apical growth after the development of the archegonia is very limited. Leitgeb (1877) stated that this is because the apical cell ceased activity, but later Renzaglia (1982) asserted that the majority of growth occurs some distance from the apex through the process of elongation. Therefore, we interpret *Noteroclada* to be acrogynous. Consequently, the lineage *Pleurozia–Noteroclada–Metzgeria* and the rest of the leafy liverworts is supported as a monophyletic group by the acrogynous condition that is a synapomorphy of all members of the group.

Another morphological character that is used to separate the simple thalloid and the leafy liverworts is the leaf initial. In the simple thalloid group, it is generally accepted that the leaf is derived from a single, centrally located cell of three-celled lateral derivatives, whereas the leaf in typical leafy liverworts is considered to derive from the two outer initials of the five-celled apical cell derivatives. However, leaf development in *Pleurozia* is different from that of the typical jungermannioid leafy liverworts. According to Renzaglia (1982), the leaf of *Pleurozia* is derived from the three outer cells of a five-celled lateral merophyte, which coincides with that of the Metzgeriidae, although Crandall-Stotler (1981) assumed that the leaf development of *Pleurozia* at a later stage is unique and highly modified. Crandall-Stotler (1976) stated that the formation of the shortly bifid lobe of *Pleurozia* resembles the leaf development of typical Jungermanniidae through a two-celled leaf initial. So far, the mechanisms of the formation of the two growing points, which later develop into lobed leaves of the Jungermanniidae, and the single growing point, which results in the unlobed leaf structure of the simple thalloid liverworts, are still not clear.

The sister relationship between *Pleurozia* and *Noteroclada* and *Metzgeria* in the present analysis suggests, however, that separating the leafy and simple thalloid liverworts by only leaf initial criterion is probably not feasible. The two-celled leaf initial might have evolved independently in *Pleurozia* and in the rest of leafy lineages, or it might have evolved once in the lineage *Pleurozia–Noteroclada–Metzgeria* and rest of the leafy liverworts, but *Metzgeria* and *Noteroclada* have lost this feature and there has been a reversal to a single leaf initial stage. In either case, the *Pleurozia–Noteroclada–Metzgeria* lineage and rest of the leafy liverworts can be considered to form a monophyletic group.

In addition to the acrogynous gametangial position in the Metzgeriaceae, Aneuraceae, and Mizutaniaceae, many other morphological features approach those found in the Jungermanniidae, such as the terminal elaterophore, the 2-layered capsule wall, the 2 to 4 valves of the capsule, and the 1 spiral elaters. Our inferences suggest that *Noteroclada* and *Metzgeria* are closely related to the leafy *Pleurozia,* and that the remaining simple thalloid and leafy liverworts form sister groups. Because of the systematic position of *Pleurozia,* the acrogynous condition and the abovementioned morphological similarities in *Metzgeria* and the Jungermanniidae, we suggest regarding the clade *Pleurozia–Metzgeria–Noteroclada* as belonging together with the rest of the leafy liverworts. The position of *Hymenophyton flabellatum* cannot be confirmed, since more than half of its molecular data are lacking in our analysis. Other acrogynous, simple thalloid genera and families were not included here, and their relationships with the leafy liverworts need to be tested in further studies.

If the acrogynous simple thalloid group is excluded, the core simple thalloid liverworts that remain are mainly characterized by the anacrogynous condition and the sex organs that are usually located on the dorsal midline of the gametophyte. According to our study, the cuneate apical cell seems to be the primitive condition in this group. Included in our analysis, *Pellia* and *Makinoa* have cuneate apical cells, and *Pellia* is the basal group of the simple thalloid liverworts in our tree (Fig. 1). However, more simple thalloid taxa should be added in future study to confirm this hypothesis.

Schuster (1992) assumed that leafiness in the simple thalloid liverworts is primitive; therefore, he proposed six leafy genera, *Treubia, Phyllothallia* E. A. Hodgs., *Noteroclada, Symphyogyna* Nees &

Mont., and *Fossombronia*, as separate starting points of evolution. Although not all of the above genera are included in our analyses, the results do not support this viewpoint. According to our results, the thalloid gametophyte may be the original form, and leafy forms such as *Fossombronia* and *Haplomitrium* are more derived.

Haplomitrium is placed in the simple thalloid clade in the most congruent topology, but its position changes when using different optimization parameters. Morphologically, *Haplomitrium* is unique in that it resembles the simple thalloid liverworts in its anacrogynous condition and in the presence of a vascular system (Schuster, 1984a), but it differs markedly from the simple thalloid liverworts by its tetrahedral apical cell with three equally cutting faces, its lack of rhizoids, its identical early gametangium development, and its one-layered sporophyte wall. Schuster treats *Haplomitrium* as the basal most genus among the whole liverworts, and claims (Schuster, 2000) that the genus has numerous plesiomorphic features, for example, the lack of a sporophyte protecting structure, a triradial gametophyte, anacrogynous gynoecia, the unstructured dehiscence of the capsule, and the relatively massive spermatozoid. However, our study does not recognize those features as plesiomorphies. We consider the triradial organization to be a derived feature. In Crandall-Stotler and Stotler's (2000) trees, *Haplomitrium* belongs to a lineage that is sister to the clade consisting of *Fossombronia* and all the leafy liverworts, yet they placed it at the beginning of the Metzgeriidae in their classification. It seems that the position of *Haplomitrium* is still ambiguous, and more characters should be further investigated.

ON LEAFY LIVERWORTS

Since the leafy liverworts represent the majority of liverwort species and their phylogenetic relationships are least explored, we sampled the leafy liverworts comprehensively, i.e., most of the suborders defined by Schuster (1972) are included. In addition, we tested Schuster's theory on the origins and evolution of the leafy liverworts and his classification at three levels: species that make up the genera, genera that constitute the families, and families that are said to be closely related in suborders.

The leafy liverworts in the study are resolved as a monophyletic group, formed by two sister clades, the clade *Pleurozia–Noteroclada–Metzgeria* and the clade that consists of the rest of the leafy liverworts. The latter clade is further divided into two sister groups, which we refer to as the Porellales–Lepidolaenineae clade (from *Porella* to *Cololejeunea*), and the Perssoniellineae–Herbertineae clade (from *Schistochila* to *Herbertus*). The reconstruction of two major evolutionary lines is contrary to Schuster's (1996, 1972, 1979) theory that a number of suborders radiated suddenly just after the leafy liverworts diverged from the rest of the land plants.

Clearly, the two major groups of the leafy liverworts have evolved unequally in numbers of genera. Although the acrogynous condition supports recognition of the group *Pleurozia–Noteroclada–Metzgeria* with the Bremer support value (BS = 29, Fig. 1), their morphology differs remarkably. *Noteroclada* and *Metzgeria* still retain some simple thalloid features, such as the thalloid plant body, non-localized rhizoids, and scattered antheridia and elaters attached at the foot of the capsule in *Noteroclada*. The tetrahedral apical cell seen in *Noteroclada* and the rest of the leafy liverworts appears to be the original apical cell type for the whole leafy liverwort clade. Unlike the dorsiventral growth form of *Noteroclada* and *Metzgeria*, *Pleurozia* has evolved the erect growth form, comparable to many other jungermannioid species. Schuster (1965) proposed that *Pleurozia* should be placed after the suborder Jungermanniineae because its scattered rhizoids, its succubous leaf insertion, and its succubous-complicate leaves resemble those found in the Scapaniaceae Mig., Balantiopsidaceae H. Buch, and Delavayellaceae R. M. Schust. However, *Pleurozia* has always been positioned last as suborder or order in various subsequent classification schemes (Schuster, 1966, 1972; Schljakov, 1972; Crandall-Stotler & Stotler, 2000). This treatment was partially based on a commonly accepted interpretation that the lenticular apical cell is an advanced condition (Schuster, 1965) or highly modified (Crandall-Stotler, 1981). Our results suggest that *Pleurozia* diverged early after the split of the two leafy groups and should fall at the beginning of the leafy liverworts in any classification.

Compared with the *Pleurozia–Noteroclada–Metzgeria* clade, the clades Porellales–Lepidolaenineae and Perssoniellineae–Herbertineae are highly diverse in terms of species numbers and morphological characters. Morphologically, the tetrahedral apical cell with three derivatives is found throughout this large group, and it seems that most probably this kind of apical cell, which leads to an erect growth form (see Crandall-Stotler, 1981), plays an important role in the success of speciation and evolution. The derivation of the triradial symmetry with three rows of similar or identical leaves is likely dictated by the apical cell. The growth of the ma-

jority of the mosses and vascular plants is also initiated by a tetrahedral apical cell.

Throughout the leafy liverworts, bilateral and anisophyllous organization and lateral branching are present, but triradial and isophyllous organization and evolving of ventral branching are only present in the clade Perssoniellineae–Herbertineae. Because the features of isophylly and ventral branching are lacking in the Porellales–Lepidolaenineae clade and the Schistochilaceae H. Buch, it probably indicates that these characters evolved later. This result is contrary to Schuster's theory that ancestral gametophytes of the leafy liverworts were not only erect and isophyllous, but their branches issuing from all three merophyte rows (Schuster, 1996a).

ON THE PORELLALES–LEPIDOLAENINEAE CLADE

This clade contains the families that have been assigned to the order Porellales (Schljakov, 1972; Crandall-Stotler & Stotler, 2000; Schuster, 1953 as suborder Porellineae R. M. Schust.) and to the suborder Lepidolaenineae (Schuster, 1972; Crandall-Stotler & Stotler, 2000). In addition, genera *Ptilidium* and *Radula* from the orders Ptilidiales and Radulales (Schljakov, 1972; Crandall-Stotler & Stotler, 2000; Schuster, 1953 and 1958 as suborders Radulineae R. M. Schust. and Ptilidiineae) are found in this clade. The clade is quite well supported (BS = 24, Fig. 1). In general, the composition and taxonomic relationships of this clade are very similar to the results of Ahonen (2004), except for the presence of Lepidolaenaceae Nakai, *Goebeliella,* and *Neotrichocolea* S. Hatt., which were not present in that study. The surprising position of *Ptilidium pulcherrimum* within Porellales in Ahonen (2004) is confirmed here.

In Schuster's (1980) classification system, Ptilidiineae and Lepidolaenineae were placed just below the Porellineae, and he postulated that they could represent an ancestral form of the Porellales (Schuster, 1966, 1984a, 1992). Crandall-Stotler and Stotler (2000), however, placed the Ptilidiales and Lepidolaenineae (Lepicoleales) in the beginning of their classification system of the leafy liverworts, while the order Porellales was at the end of the system. Schuster (1953) first placed the family Radulaceae in the Porellales, but later removed it to a suborder of its own taxonomically close to the Porellineae. Crandall-Stotler and Stotler (2000) followed this, only elevating the suborder to the order Radulales.

The Porellales–Lepidolaenineae–*Radula*–*Ptilidium* clade shares many morphological characters that make this grouping plausible. The most obvious is the presence of a lobule of some kind in every member of the clade except *Ptilidium.* The lobule is the ventral lobe of the leaf that is folded against the dorsal lobe and often forms a water sac. There are five types of lobules in this clade, Porellaceae Cavers, Radulaceae (Dumort.) Müll. Frib., Goebeliellaceae Verd., Lejeuneaceae, and Frullaniaceae Lorch. types. The Frullaniaceae type of lobule, a galeate, helmet-shaped lobule formed by the central cells of the leaf lobe dividing faster than the marginal cells, seems to be the most widespread. In addition to genera *Frullania* and *Jubula* (and some small genera that were not included in this study, like *Neohattoria* Kamim., *Schusterella* S. Hatt., and *Amphijubula* R. M. Schust.), it is found also in all Lepidolaenineae, except the genus *Trichocoleopsis* S. Okamura, which was not included in this study. Galeate lobule could represent the ancestral lobule type of this clade.

An impressive list of other synapomorphies are found in this clade: *Frullania*-type branching, rhizoids restricted to the underleaf bases, cortical cells differentiated to thickened outer cells and thin-walled medullary cells, the presence of a stylus, small homogenous oil bodies, reddish and brownish pigments, *Frullania*-type germination pattern, broad ventral merophytes, endosporic germination, and asexual reproduction via discoid gemmae or caducous leaves. However, none of these characters is found throughout the clade, the most deviating genus being *Radula,* which differs from the other plants in almost every feature mentioned above. Even *Ptilidium* fails to show the *Frullania*-type sporeling, endosporic germination, a stylus, asexual reproduction, or the presence of a lobule. Ciliate leaves are found in many (e.g., Lepidolaenineae, *Jubula, Ptilidium*), but not all, members of the clade.

All species of this clade share anisophyllous leaves, incubous leaf orientation, and epiphytism. These could well represent the ancestral states, not only of this clade, but of all the leafy liverworts. We suggest this because *Schistochila* Dumort., *Paraschistochila,* and *Gottschea* Nees ex Mont., which are basal in the Perssoniellineae–Herbertinae clade, exhibit these same features.

However, all of the above features are also found occasionally among the other leafy liverworts and are probably plesiomorphic characters. Good candidates for a true synapomorphy are the pinguisane-type sesquiterpenes that have been found in leafy liverworts only in Ptilidiaceae H. Klinggr., Lepidolaenaceae, Porellaceae, and Lejeuneaceae (Asakawa, 1982).

The clade is divided in two sister groups, one

with the Lejeuneaceae and the *Jubula* and *Nipponolejeunea*, the other with the Lepidolaenineae, *Goebeliella*, *Frullania*, *Ptilidium*, and *Radula*. The well-supported (BS = 41, Fig. 1) Lejeuneaceae–*Jubula* clade confirms the views of Mizutani (1961) and Ahonen (2004) that the family Lejeuneaceae and the genus *Jubula* are taxonomically very close. This grouping can also be seen in Gradstein et al. (2003). *Nipponolejeunea* groups strongly with *Jubula*, rather than with the Lejeuneaceae, in accordance with Mizutani (1961), Ahonen et al. (2003), Ahonen (2004), and Wilson & Gradstein (2003), but not with Weis (2001) or Gradstein et al. (2003).

The synapomorphies of the *Porella–Frullania–Radula–Ptilidium*–Lepidolaenineae clade are several archegonia per perianth and free elaters in all the taxa except the genus *Frullania*. However, the Bremer support for this clade is not very high (BS = 8, Fig. 1), and even the two sister clades it contains, the *Porella–Radula–Frullania* clade and the *Ptilidium–Goebeliella*–Lepidolaenineae clade, have only low support values (BS = 3 and BS = 8, respectively, Fig. 1). Thus, the present tree may not accurately reflect the relationships of the subgroups of this clade. Perhaps the addition of intermediate genera, such as *Amphijubula Neohattoria*, *Schusterella*, and *Steerea* S. Hatt. & Kamim. (intermediate between *Frullania* and *Jubula*), would further elucidate the true relationships in this clade.

The only common feature of the *Porella–Frullania–Radula* clade is the variable chromosome number in all these genera, while the other Porellales and Lepidolaenineae always have the chromosome number nine (Fritsch, 1991; Ahonen, 2004). Morphologically, *Porella*, *Radula*, and *Frullania* are rather different from each other. In accordance with Ahonen (2004), *Frullania* groups with *Radula* rather than *Jubula*, with which it has been traditionally placed in the same family (e.g., Lorch, 1914; Casares-Gil, 1919; Kamimura, 1961; Schuster, 1992; Crandall-Stotler & Stotler, 2000). *Frullania* and *Radula* group very strongly together (BS = 36, Fig. 1). However, the branches of both *Radula* and *Frullania* are very long (data not shown) indicating a possibility of long branch attraction, so they need more focused studies.

Members of the *Ptilidium–Goebeliella*–Lepidolaenineae clade lack asexual reproduction, and all members except *Goebeliella* have ciliate leaves divided to many lobes. All members except *Ptilidium* and *Goebeliella* have galeate lobules. Members of the Lepidolaenineae have a coleocaule instead of a perianth. This is why the Lepidolaenineae have been considered more primitive than the Porellales, despite the very similar lobule in both groups. However, the coleocaule appears to be a derived feature, at least in this clade.

Neotrichocolea and *Ptilidium* group strongly together (BS = 30, Fig. 1), as a sister group to the *Goebeliella*–Lepidolaenaceae–*Jubulopsis* clade. *Neotrichocolea* and *Ptilidium* look superficially similar based on their ciliate leaves, but differ profoundly in such features as the presence of a coleocaule, endosporic germination, and subfloral innovations in *Neotrichocolea*. Moreover, *Ptilidium* lacks lobules. This could be due to *Ptilidium* having lost the lobule, rather than the more traditional explanation that the lobulate leaf has developed from the *Ptilidium*-type leaf (Schuster, 1984). The addition of *Trichocoleopsis* S. Okamura may clarify this part of the tree, because it is supposed to be taxonomically close to either *Neotrichocolea* (Schuster, 1972) or Lepidolaenaceae (Crandall-Stotler & Stotler, 2000), while it has Lejeuneaceae-type lobules.

Goebeliella is a sister to the clade containing the family Lepidolaenaceae and *Jubulopsis novae-zealandiae* from the monotypic family Jubulopsidaceae, also from the suborder Lepidolaenineae. The position of *Goebeliella* calls for a closer scrutiny, as it differs from the neighboring taxa in the tree by having a perianth, unlobed leaves, and very different lobules. *Goebeliella* has two lobules per leaf, and these are narrow and hornlike, having an opening in the upper end, while in the galeate lobule the opening points downward. In addition, the node connecting *Goebeliella* and Lepidolaenaceae has only a moderate support (BS = 12, Fig. 1). In Gradstein et al. (2003) *Goebeliella* groups with the outgroup *Porella*.

The family Lepidolaenaceae and *Jubulopsis novae-zealandiae* compose a rather well-supported clade (BS = 18, Fig. 1). All members of this group have ciliate, many-lobed leaves, galeate lobules, a coelocaule (although *Gackstroemia magellanica* also has a reduced perianth), and a stylus that is often rather large. Hamlin (1973) placed *Jubulopsis* R. M. Schust. in the Lepidolaenaceae as a subfamily Jubulopsidoideae. It is surprising that in our results *Jubulopsis novae-zealandiae* lies within *Lepidolaena*. However, *Jubulopsis* shares a scaly coelocaule and quadrifid underleaves with *Lepidolaena*, but lacks water-sacs on the underleaves, the special feature of the family Lepidolaenaceae.

Jubulopsis has been variously placed in the family Frullaniaceae (Schuster, 1970, based on the forms of the lobule and the stylus, and Hattori, 1972, based on the general morphology): within the genus *Jubula* (Hodgson, 1965); in *Neohattoria* (Grolle, 1966); and within the Lepidolaenineae

(Hamlin, 1973, based on the branching pattern, quadrate underleaves, and ciliate leaves). Later, when it was found to have a coelocaule, Schuster (1996b) placed it within the Lepidolaenineae in a family of its own, Jubulopsidaceae (Hamlin) R. M. Schust. However, all the above researchers have drawn attention to the seemingly intermediate position of *Jubulopsis* between the Lepidolaenineae and Porellales. The present result unites these views, showing that the Porellales, *Jubulopsis*, and Lepidolaenineae form a monophyletic entity.

When did the Porellales–Lepidolaenineae–*Radula*–*Ptilidium* clade diverge? The oldest fossils from its members are about 50 million years old from genera *Frullania*, *Radula*, *Nipponolejeunea*, *Bryopteris*, *Cheilolejeunea* (Spruce) Schiffn., *Trocholejeuna* Schiffn., *Cyclolejeunea* A. Evans, and *Lejeunea* Lib. (Grolle, 1981, 1982, 1983, 1984a, b, 1993, 1998). By the time these fossils were caught in the Baltic amber, the present families had already diverged from each other, so the diversification of the clade from the other leafy liverworts had to have happened earlier. The present distribution of these plants may offer some clues. The families in this clade are either cosmopolitan (Frullaniaceae, Jubulaceae, Lejeuneaceae, Porellaceae, Ptilidiaceae, and Radulaceae) or restricted to Japan, New Caledonia, Australasia, and Patagonia. This could be a relict of a former wide distribution (Schuster, 1970). At least in the case of the genus *Nipponolejeunea*, it indeed is so, as *Nipponolejeunea* fossils are found even in Europe (Grolle, 1981), whereas today they only grow in Japan, Korea, and Sacchalin. On the other hand, it could also be that Asia and Australasia—or the corresponding Gondwanaland regions—are the origin of this clade. If we do not assume long-distance dispersal as an explanation for the distribution of the cosmopolitan families, we have to assume diversification of this clade had already taken place before the separation of the Southern Hemisphere continents in the Jurassic or Cretaceous.

Schuster considered Porellales as the "summit" of liverwort evolution, with specially the family Lejeuneaceae in a state of dynamic evolution (Schuster, 1980). Schuster is likely to be correct in his view of the dynamic state of evolution of the Lejeuneaceae, but the theory that the Porellales–Lepidolaenineae–*Radula*–*Ptilidium* clade has evolved relatively recently is not supported by our results. On the contrary, the relative morphological uniformity of this clade compared to that of the jungermannioid clade indicates that it has retained various early evolved features, which are also present in the *Schistochila* lineage, such as the branching from only lateral merophytes and the incubous leaf insertion.

ON THE PERSSONIELLINEAE–HERBERTINEAE CLADE

This monophyletic clade includes many of Schuster's suborders, Perssoniellineae (Schistochilaceae), Herbertineae (Pseudolepicoleaceae Fulford & J. Taylor, Trichotemnomaceae R. M. Schust., and Herbertaceae Müll. Frib. ex Fulford & Hatcher), Balantiopsidineae (Balantiopsidaceae and Isotachidaceae), Jungermanniineae (Jungermanniaceae Rchb., Gymnomitriaceae H. Klinggr., and Scapaniaceae), Lepidoziineae (Calypogeiaceae (Müll. Frib.) Arnell, and Lepidoziaceae Limpr.), Cephaloziineae (Adelanthaceae (Jörg.) Grolle and Cephaloziaceae Mig.), Ptilidiineae (Chaetophyllopsidaceae R. M. Schust. and Mastigophoraceae R. M. Schust.), Lepidolaenineae (Trichocoleaceae Nakai), Geocalycineae R. M. Schust. (Geocalycaceae H. Klinggr. and Plagiochilaceae (Jörg.) Müll. Frib. & Herzog), and Lepicoleineae (Vetaformaceae Fulford & J. Taylor and Lepicoleaceae R. M. Schust.). The clade is well supported (BS = 41, Fig. 1), except the *Schistochila*–*Paraschistochila* assemblage (BS = 7, Fig. 1), of which Schistochilaceae clustered within the Porellales–Lepidolaenineae clade when using only *rps*4 data (tree not shown).

No clear morphological synapomorphies are found in the Perssoniellineae–Herbertineae clade, but, except for the *Schistochila*–*Paraschistochila* assemblage, the clade is distinctive by its branches arising from all three merophytes and its succubous leaf insertion. Many groupings obtained here are in agreement with the traditional arrangements at different taxonomic levels. For example, the Schistochilaceae is monophyletic with the genera *Schistochila*, *Gottschea*, and *Parachistochila* (BS = 53, Fig. 1); *Odontoschistoma* (Dumort.) Dumort., *Schiffneria* Steph., *Cephalozia* (Dumort.) Dumort., and *Nowellia* Mitt. from the Cephaloziaceae group together (BS = 19, Fig. 1); *Zoopsis* Hook. f. ex Gottsche, Lindenb. & Nees, *Hygrolembidium* R. M. Schust., *Bazzania* Gray, and *Lepidozia* (Dumort.) Dumort. from the Lepidoziaceae (BS = 25, Fig. 1) form another well-supported clade. The families Plagiochilaceae and Geocalycaceae group together and give support to the existence of the suborder Geocalycineae (BS = 23, Fig. 1); Vetaformaceae and Lepicoleaceae, as well as the closely related *Triandrophyllum* Fulford & Hatcher and *Herbertus* Gray group together to give support to the existence of the Lepicoleineae (BS = 26, Fig. 1) and the family Herbertaceae (BS = 12, Fig. 1).

The monophyletic Schistochilaceae is resolved

herein as the sister group to the remainder of the clade (Fig. 1). This hypothesis is, however, weakly supported. Members of the Schistochilaceae are morphologically similar to those of the Porellales–Lepidolaenineae clade. For example, they share the following characters: the gametophytes are dorsiventrally compressed, the leaves are incubous, branches arise only from lateral merophytes, the ventral merophytes are broad, and the leaf margins are ciliate. The Schistochilaceae also has unique features, such as the linear but erect capsule valves, two types of rhizoids, rhizoids branched and septate at the apices, and numerous antheridia per male bract (Schuster & Engel, 1977). Schuster (1964b) postulated that the Schistochilaceae (Perssoniellineae) has no close affinities with any other leafy liverworts, but he (1984a) placed the suborder between the Geocalycineae and Radulineae, and later between Brevianthineae J. J. Engel & R. M. Schust. and Balantiopsidineae (Schuster, 2000). Crandall-Stotler and Stotler (2000), by contrast, placed it at the beginning of the leafy liverworts, within the order Lepicoleales Stotler & Crand.-Stotl. Our study suggests that the Schistochilaceae (represented herein by *Schistochila appendiculata, S. laminigera, Gottschea nuda,* and *Parachistochila tuloides*) alone diverged very early after the split of the Porellales–Lepidolaenineae clade and the Perssoniellineae–Herbertineae clade. It is not closely related to the Radulineae, and only remotely related to the Geocalycineae, Balantiopsidineae, and Lepicoleales sensu Stotler and Crand.-Stotl.

Schuster (1972, 2000) placed the Lepicoleineae and the Herbertineae at the beginning in his classification system, asserting that the triradial and isophyllous gametophytes, variable branching patterns, and lack of perianth are primitive features. Our study does not support this viewpoint, but rather suggests that the branching from both lateral and ventral merophytes in the suborders Lepicoleineae, Herbertineae, Balantiopsidineae, Jungermanniineae, Cephaloziineae, Lepidolaenineae, and Geocalycineae is a derived feature. Moreover, protection of the developing sporophytes by a coelocaule, not by a perianth, is present in various lineages, as in Lepidolaenineae, Perssoniellineae, Lepicoleineae, and Ptilidiineae. Therefore, coelocaule formation has likely evolved several times independently. The perianth has evolved in *Pleurozia* and in other the two major leafy groups of the Porellales–Lepidolaenineae and the Perssoniellineae–Herbertineae. Lack of a perianth, therefore, could not be regarded as a primitive feature.

The clade *Blepharostoma–Isotachis* represents the first lineage that has evolved branching from both lateral and ventral merophytes; *Blepharostoma* (Dumort.) Dumort. might have been the original type for the lateral-ventral branching group. The sister group of the clade has further evolved into two lines, referred to here as the Jungermanniineae–Cephaloziineae clade and the Lepidoziineae–Geocalycineae–Lepicoleineae–Herbertineae clade. Our results suggest that the suborders Jungermanniineae and Cephaloziineae are closely related and the Lepidoziineae, Geocalycineae, and Herbertineae are allies. These groupings are also seen in classifications based on morphology. For instance, Schuster (1969) treats the Jungermanniineae as including the Cephaloziaceae s.l. In other classification schemes (Schuster, 1972; Schljakov, 1972; Crandall-Stotler & Stotler, 2000) the Cephaloziineae and Jungermanniineae are placed close to each other. Morphologically, the Cephaloziineae and Jungermanniineae share many features, such as the scattered rhizoids and the 1–2-celled gemmae as the dominant form of asexual reproduction. Our study supports uniting the Cephaloziineae and the Jungermanniineae as a single suborder because species of these two groups are greatly mixed in the topology (see Lophoziaceae, Cephaloziaceae and *Nardia assamica* of the Jungermanniaceae); but, more taxa from the Jungermanniaceae, Cephaloziellaceae and Adelanthaceae should be added to test our result. However, the commonly accepted opinion that the Cephaloziineae is derived from the Lepidoziineae (Schuster, 1972) is not supported, because the study shows that the Cephaloziineae and the Lepidoziineae have evolved from two different lines as shown here, and their morphology differs fundamentally in leaf form, rhizoid distribution pattern, and the number of epidermal cells in the seta.

The Lepicoleineae, Herbertineae, and Lepidoziineae have been linked together in various classification systems (Grolle, 1983; Schuster, 2000). However, the connection of the Geocalycineae to the above three suborders was not addressed in previous studies. Morphological characters that are common in all these taxa are rhizoids restricted to the underleaf base, gametangia on leading leafy axes, isophyllous female bracts, and a trigonous perianth. The systematic relationships among the four suborders should be thoroughly studied.

Blepharostoma from the Pseudolepicoleaceae (Herbertineae) and the Trichotemnomaceae group together with the Balantiopsidaceae, far from the rest of the suborder. The Herbertaceae is the sister group of the Mastigophoraceae, and *Temnoma* Mitt. of the Pseudolepicoleaceae is placed with the Lepidoziaceae. If future studies confirm the position of

the Pseudolepicoleaceae and Trichotemnomaceae, then the *Blepharostoma–Isotachis* clade should be proposed as an independent suborder to include the Pseudolepicoleaceae, Trichotemnomaceae, and the current Balantiopsidineae. The close relationship between the Herbertaceae and Mastigophoraceae in the study supports Grolle's treatment of the Mastigophoraceae as a subfamily in the Lepicoleaceae (Grolle, 1983), and not Schuster's concept (1987) that it belonged in the Ptilidiineae. The position of *Temnoma* within the Lepidoziineae and the Trichocoleaceae within the Geocalycineae suggests that the paraphyletic Herbertineae should be further investigated.

Chaetophyllopsis whiteleggei of the Ptilidiineae is nested within the Jungermanniineae (BS = 22, Fig. 1). Schuster (1960) assigned the family Chaetophyllopsidaceae to the Ptilidiineae on the basis of shared characters with Ptilidiaceae, such as the pinnate branching pattern, the asymmetrically lobed leaves, the ciliate leaf margins, and the presence of perianth. However, as stated by Schuster, the family is also marked by a series of critical differences from the rest of the Ptilidiineae, for instance, the succubous leaf insertion, the soft-textured stem, and the presence of pluricellular gemmae. These distinctive features of Chaetophyllopsidaceae fit well in the Jungermanniineae, and our study suggests removing the family Chaetophyllopsidaceae from the Ptilidiineae and placing it in the Jungermanniineae.

In the clade Jungermanniineae–Cephaloziineae, the family Scapaniaceae is mixed among the family Lophoziaceae, which suggests that boundaries between the two groups may be weak. The systematic position of *Adelanthus lindenbergianus* and *Calypogeia muelleriana* cannot be confirmed because their sequences were incomplete. The Calypogeiaceae is usually placed in the Lepidoziineae (Schuster, 1966, 1972; Schljakov, 1972; Crandall-Stotler & Stotler, 2000), but our study shows that it is closely related to the Jungermanniineae. The Calypogeiaceae differ markedly from the Lepidoziineae by bearing a pendent marsupium. *Pedinophyllum truncatum* of the Plagiochilaceae is placed within the Jungermanniineae, not placed next to *Plagiochila*. The morphology of the genus does indeed differ from *Plagiochila* in that it has entire leaves, the stem cortical cells are not differentiated from the medullary cells, and the leaves have almost a straight insertion line. Buch (1936) placed *Pedinophyllum* in the Jungermanniaceae, and Schuster (1980) stated that the genus could be placed with some justification into either the Jungermanniaceae or Plagiochilaceae. Our study supports the former placement.

In the present study, the gametophytes of *Schiffneria hyalina* of the Cephaloziaceae and *Zoopsis argentea* of the Lepidoziaceae are semi-thalloid, remarkably different from other members of their respective families. The monophyly of the two families indicates that the thalloid growth form is secondary in these cases.

Our study shows that the leafy liverworts, which have branches developing from both lateral and ventral merophytes, have evolved three times: in the *Blepharostoma–Isotachis* clade, in the Jungermanniineae–Cephaloziineae clade, and in the Lepidoziineae–Geocalycineae–Lepicoleineae–Herbertineae clade. If further study confirms this result, it would mean that this large, diverse group can be reorganized in a sound phylogenetic order. Such a reordering would provide a framework for re-evaluation of morphological characters of the group.

Conclusions

Our study suggests that liverworts are a monophyletic plant group, and it is the earliest lineage of land plants. We conclude that *Blasia pusilla* should be accommodated its own class, the Blasiopsida, as it is sister to the rest of the liverwort species. The ancestral type of liverworts is likely to have been a simple thalloid gametophyte, with growth via a cuneate apical cell with four cutting faces and bearing a sporophyte with massive foot and seta. The complex thalloid liverworts are monophyletic and a sister group to the simple thalloid and leafy liverworts. The complex thalloid and simple thalloid/leafy liverworts are separate lineages. The concept of the simple thalloid liverworts is redefined in terms of our suggestion of removing the acrogynous taxa (*Metzgeria* and *Noteroclada*) to the jungermannioid leafy liverwort group. This would leave the remaining simple thalloid liverworts as a monophyletic group. The leafy liverworts are resolved as a monophyletic group formed by two sister clades, the clade *Pleurozia–Noteroclada–Metzgeria* and the clade which is further divided into two sister groups, the Porellales–Lepidolaenineae clade and the Perssoniellineae–Herbertineae clade. The sister relationship between *Pleurozia* and *Noteroclada* and *Metzgeria* suggests that separating the leafy and simple thalloid liverworts by only leaf initial criterion is probably not feasible. The bilateral and anisophyllous gametophyte organization and branches arising from only lateral merophytes mostly present in the Porellales–Lepidolaenineae clade are suggested to be original, whereas the tri-

radial and isophyllous organization and branching from both lateral and ventral merophytes of the most of the Perssoniellineae–Herbertineae clade, derived. The leafy liverworts that have branching from both lateral and ventral merophytes have evolved in three evolutionary lines: the *Blepharostoma–Isotachis* clade, the Jungermanniineae–Cephaloziineae clade, and the Lepidoziineae–Geocalycineae–Lepicoleineae–Herbertineae clade. The tetrahedral apical cell with three derivatives is found in the majority of the leafy liverworts, and it seems that this kind of apical cell has played an important role in the evolutionary success of those liverworts.

The use of multiple nucleotide sequence data with a wide range of taxon selection in the present study has led to the discovery of many previously unsuspected relationships in the liverworts, for example, that the evolution of the liverworts have proceeded from a thalloid form to a leafy form and that the leafy liverworts are divided into two sister clades instead of the bush-like phylogeny proposed by Schuster (1979). Our study has also confirmed many existing hypotheses on relationships in the liverworts that were based on morphological investigations. An example of this is the distant relationship between the complex thalloid liverworts and the simple thalloid and leafy liverworts. It has improved our understanding of the early evolution of the land plants by showing the relationship of the liverworts to the other land plants, by revealing the characteristics of the earliest liverworts, and how the diversity of forms arose at an early stage. It suggests that many morphological and ontogenetic characters should be re-evaluated and more carefully studied. For instance, the strict division of the leafy and thalloid taxa according to the number of merophyte cells should be re-evaluated. We hope that the results of the present work will provide a framework for a sound classification of liverworts in the future. The phylogenetic hypotheses generated here should be further tested by adding morphology and by the addition of more genomic data.

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A PHYLOGENY OF THE SIMPLE THALLOID LIVERWORTS (JUNGERMANNIOPSIDA, METZGERIIDAE) AS INFERRED FROM FIVE CHLOROPLAST GENES[1]

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Abstract

The Metzgeriidae, as defined by Crandall-Stotler and Stotler (2000), comprise 38 genera, distributed in 15 families and five orders. There is substantial morphological heterogeneity within the group, including four distinct types of apical cell geometries, both leafy and thalloid organizations, development of four distinct types of hydrolyzed strand cells, and three different patterns of capsule dehiscence. This group has traditionally been viewed as pivotal in liverwort phylogeny, with some workers viewing the Metzgeriidae as basal to both the leafy and complex thalloid lineages, and others viewing it as a polyphyletic assemblage of derived lineages. To resolve the phylogenetic history of the group and test current hypotheses of intergeneric relationships, DNA sequence data were gathered for five plastid loci, namely, *atp*B, *psb*A, *rbc*L, *rps*4, and *trn*L, for 24 genera of simple thalloid liverworts plus 11 taxa of the Jungermanniideae and six representatives of the Marchantiopsida. Analyses resolve 21 of the 24 genera treated in the Metzgeriidae in a monophyletic assemblage that also includes the leafy liverwort lineage. *Blasia* is resolved, with strong support, as sister to the complex thalloid clade, and *Treubia* is resolved as sister to all other liverworts. The position of *Haplomitrium* is equivocal, either resolving within the main simple thalloid clade, or as sister to *Treubia*.

Key words: *atp*B, Metzgeriidae, *psb*A, *rbc*L, *rps*4, simple thalloid liverworts, *trn*L.

Traditionally, two morphologically defined classes are recognized in liverwort classification, namely, the Marchantiopsida Stotler & Crand.-Stotl., or complex thalloid lineage, and the Jungermanniopsida Stotler & Crand.-Stotl., or leafy/simple thalloid lineage (Crandall-Stotler & Stotler, 2000). The Metzgeriidae Barthol.-Began, as defined by Crandall-Stotler and Stotler (2000), comprise those taxa of the Jungermanniopsida that form leaves or thallus wings from single wedge-shaped initials (Renzaglia, 1982) and are fundamentally anacrogynous. Of the estimated 6000 to 8000 species of liverworts, fewer than 500 are distributed among the 38 genera and 15 families of the Metzgeriidae, and approximately 350 comprise the Marchantiopsida. With a fossil record that dates to the late Devonian (Krassilov & Schuster, 1984; Oostendorp, 1987), the Metzgeriidae appear to be the most ancient group of liverworts and, as such, are pivotal to schemes of liverwort phylogeny. To date, the position of the simple thalloids in relation to the leafy and complex thalloid groups is equivocal. Some authors have postulated linear evolution from complex thalloid through simple thalloid to leafy forms (e.g., Cavers, 1911), while others have proposed that leafy liverworts were the prototype that gave rise to both simple and complex thalloid lineages (e.g., Evans, 1939). Schuster (1992: 301) contended that the simple thalloids are "polyphyletic and originated from several distinct ancestral types." Most recently, phylogenetic analyses of both morphological (Crandall-Stotler & Stotler, 2000) and molecular (Lewis et al., 1997) data sets suggest that the Metzgeriidae may comprise a basal grade to the Jungermanniopsida, whereas ultrastructural data link the simple thalloid taxon *Blasia pusilla* L. to the Marchantiopsida (Renzaglia & Duckett, 1987; Garbary et al., 1993).

Schuster (1992) hypothesized that there are at least nine phylogenetically separate groups of an-

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acrogynous liverworts, one comprising the morphologically isolated Haplomitriales H. Buch ex Schljakov, with one extant genus of seven species (Bartholomew-Began, 1991), and the other eight comprising the suborders Blasiineae R. M. Schust., Treubiineae R. M. Schust. ex Stotler & Crand.-Stotl., Fossombroniineae R. M. Schust. ex Stotler & Crandl.-Stotl., Phyllothalliineae R. M. Schust., Pelliineae R. M. Schust. ex Schljakov, Pallaviciniineae R. M. Schust., Metzgeriineae R. M. Schust. ex Schljakov, and Hymenophytineae R. M. Schust. ex Schljakov of the Metzgeriales R. M. Schust. ex Schljakov. His phylogenetic arrangement is based on the premise that the ancestral characters for each group include the following traits: nodal or "leafy" gametophyte organization; tetrahedral apical cells; vestigial conducting tissue; archegonia naked and scattered on the dorsal surface of leading axes; immense spermatids; large massive sporophytes that are protected solely by fleshy calyptrae; multilayered capsule walls with different patterns of inner and outer wall thickenings; and large, highly sculpted spores (Schuster, 1992, 1999). Within this phylogeny the Treubiineae, Phyllothallineae, and Fossombroniineae were considered to be the least derived elements of the Metzgeriales, and the Metzgeriineae and Hymenophytineae, the most derived.

Formulating hypotheses of relationships among the Metzgeriidae is confounded by the extensive morphological heterogeneity of the group. Apical cells, for example, can be tetrahedral, cuneate, hemidiscoid, or lenticular, with as many as three different types occurring within single suborders. Conducting strands, which Schuster (1992) viewed as plesiomorphic for the group, actually comprise four developmentally and structurally different types of cells (Kobiyama, 2003), which may not be phylogenetically related. Taxa in which the thallus is dissected into leaf-like appendages occur in six of the eight suborders recognized by Crandall-Stotler and Stotler (2000), and capsules with multistratose walls display three unrelated types of internal organization and dehiscence patterns. Phylogenetic analyses of morphological data sets have produced conflicting results, without strong statistical support for the backbone relationships resolved (Garbary et al., 1993; Garbary & Renzaglia, 1999; Crandall-Stotler & Stotler, 2000; Renzaglia et al., 2000).

Early molecular phylogenies of embryophytes that included liverworts have also been unsatisfactory in resolving relationships within the Metzgeriidae (e.g., Waters et al., 1992; Mishler et al., 1992, 1994; Hiesel et al., 1994; Manhart, 1994; Capesius, 1995; Kranz et al., 1995; Bopp & Capesius, 1995, 1996; Hedderson et al., 1996; Lewis et al., 1997; Capesius & Bopp, 1997; Samigullin et al., 1998; Beckert et al., 1999; Duff & Nickrent, 1999; Nishiyama & Kato, 1999). These studies did not sample well within the major groups of liverworts and were incongruent in resolving the relationships among the major liverwort clades. More recent molecular studies have either focused on the Marchantiopsida, or complex thalloid liverworts, (D. Long et al., 2000; Wheeler, 2000; Boisselier-Dubayle et al., 2002), or if broader in taxon sampling, have analyzed only the short, fast-evolving intron of the chloroplast *trn*L_{UAA} genic region (Stech et al., 2000, 2002; Stech & Frey, 2001). Even though Stech and Frey (2001) included key taxa of the Metzgeriidae in their studies, their analyses were based on only 225 bases of this intron and lack strongly supported resolution. Without a robust phylogeny for the liverworts, anatomical and biochemical studies aimed at understanding character evolution in early land plants, such as those of Kobiyama (2003), Poli et al. (2003), and Popper and Fry (2003), will be based on incomplete and often erroneous phylogenetic hypotheses.

Since the Metzgeriidae express many of the structural features postulated to be plesiomorphic in hepatics (Schuster, 1999), we have chosen to focus on this group. The objectives of this current study are to determine whether the Metzgeriidae, as currently defined, are monophyletic; to resolve the position of the Metzgeriidae within the Marchantiophyta; and to identify the major phylogenetic lineages within the subclass. We have focused on five regions of the chloroplast genome, which vary both in length and substitution rate. Four of these are protein-coding genes. These are *rbc*L, which codes for the large subunit protein of the photosynthetic enzyme ribulose bisphosphate carboxylase/oxygenase (Olmstead & Palmer, 1994), *rps*4, which codes for protein 4 of the chloroplast small ribosomal subunit (Nadot et al., 1994), *psb*A, which codes for the D1 core protein of photosystem II (Morton & Levin, 1997; Jobes et al., 1998), and *atp*B, which codes the B subunit of ATP synthase (Hoot et al., 1995). The fifth region encompasses the $tRNA_{leu}$ intron and 3′ exon and the *trn*L/*trn*F intergenic spacer, included between the universal *trn*L/*trn*F primers c and f (Taberlet et al., 1991).

Materials and Methods

Taxon Sampling

Sequences were generated for 48 species of hepatics, representing all 8 suborders of the simple

thalloids, 6 of the 16 suborders of the leafy liverworts, and 5 of the 8 suborders of the complex thalloids. Analyses were rooted on 5 hornwort and 7 moss species, using sequences mostly obtained from GenBank. For GenBank numbers and voucher information, see Table 1.

DNA was extracted from living material, typically using 0.05–0.1 g of fresh material. Twenty-five of the 48 taxa were extracted from axenic cultures, established and maintained at Southern Illinois University, and 16 were personally collected by C-S in New Zealand in January 2002. The remaining seven taxa were collected by either students or colleagues as indicated in Table 1. Tissue was ground in an Eppendorf tube, using a plastic pestle and a pinch of sea-washed sand. Most DNA extractions were done using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, U.S.A.), with the first elution in 50 μl buffer AE and a second elution in 75 μl of the same buffer. Some taxa were amplified using the protocol in Bray and Scott (2000), with a final resuspension in 25 μl nanopure water.

PCR METHODS

Polymerase chain reaction (PCR) was performed in 50 μl reactions (1.5 units Promega *Taq* DNA polymerase (storage buffer A, Promega, Madison, Wisconsin, U.S.A.); 1 × buffer; 2.5 mM $MgCl_2$; 200 μM dNTPs (Roche Diagnostics, Basel, Switzerland); 300 μM of each primer; water to 50 μl and 0.5–2 μl of template DNA). Where the Bray and Scott (2000) procedure was used, 0.3 μl of template DNA was optimal. Reactions were run in a PTC-100 Peltier Thermal Cycler (M. J. Research, Waltham, Massachusetts, U.S.A.) employing 1 min. at 94°; then 35 cycles of 1 min. at 93°, 1 min. at 50°, 3 mins. at 72°; with a final extension step of 7 mins. at 72°; hold at 4°. The resulting double-stranded DNA amplification products were cleaned using QIAquick PCR Purification Kit and eluted in 30 μl buffer EB. The following primer combinations were used: *rbc*L—M007 or NM34 (forward), MtmRR or M636 (reverse); *rps*4—rps5′ (forward), trnAS (reverse); *psb*A—501F (forward), trnHR (reverse); *atp*B—1163F (forward), 910R (reverse); *trn*L—trnL/trnF primer C (forward), primer F (reverse). All primer sequences are detailed in Table 2.

SEQUENCING REACTIONS

Sequencing reactions were prepared using the ABI BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, U.S.A.), following standard kit protocol. The sequencing PCRs were run in accordance with the manufacturer's instructions, using some or all of the following primers in addition to the PCR primers (Table 2): *rbc*L—M985 (forward), M745 (reverse); *psb*A—trnK2F (forward), 576R (reverse); *atp*B—672F or LWT3F (forward), 384R or LWT3R (reverse); *trn*L—trnL FOSS or trnL LWT (reverse). Sequencing reactions were cleaned using Princeton Separations CentriSep Spin columns filled with 850 μl Sephadex (Sephadex G-50 fine DNA grade) hydrated in nanopure water. Sequence products were resolved on an ABI PRISM 377 Automated Sequencer.

Electropherograms were edited and forward and reverse sequences were assembled for each DNA region using Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, Michigan, U.S.A.). Sequences were aligned in PAUP* 4.0b10 (Swofford, 2002). Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2002), mounted on an Apple Macintosh G4. Regions of ambiguous alignment and incomplete data (i.e., the beginnings and ends of sequenced regions) were identified and excluded from further analyses. The g_1 statistic, which tests for the presence of significant phylogenetic signal within the data, was estimated from a random sample of 100,000 trees.

DATA ANALYSIS

Maximum parsimony (MP) analyses were run in PAUP* under Fitch parsimony, using 1000 random addition replicates, with TBR, saving 15 trees per replicate. The most parsimonious trees (MPTs) found were then input to a second round of TBR, with no limit on the number of trees saved. Bootstrapping was performed with 1000 replicates, using heuristic search strategy and simple addition, saving only one tree per replicate. This strategy was used for (a) the combined data set, including all taxa, (b) the combined data set with the two *Haplomitrium* Nees species excluded, and (c) the combined data set with the two *Treubia* K. I. Goebel species excluded. Individual MP searches were also run separately for each of the five genes with all taxa included.

MrModeltest 1.1b (Nylander, 2002) was used to establish the model of DNA evolution with the best fit to the data for the combined chloroplast DNA matrix. Maximum likelihood (ML) analyses were conducted in PAUP* (Swofford, 2002), using the starting trees defined by MP analysis, with TBR swapping. Analyses were performed using the General Time Reversible model of nucleotide substi-

Table 1. Voucher information and GenBank accession numbers for taxa sequenced in this study. All voucher specimens deposited in ABSH. "Ax" = axenic culture.

Taxon	Voucher	GenBank accession no.				
		*rbc*L	*rps*4	*trn*L	*atp*B	*psb*A
Ingroup Taxa						
Allisonia cockaynii (Steph.) R. M. Schust.	New Zealand, *Stotler & Crandall-Stotler 4470*	AY507389	AY507432	AY507518	AY507344	AY507472
Anastrophyllum michauxii (F. Weber) H. Buch	U.S.A., North Carolina, *Sargent s.n.* (Ax)	AY507390	AY507433	AY507519	AY507345	AY507473
Aneura pinguis (L.) Dumort.	U.S.A., Wisconsin, *Hatcher culture #12* (Ax)	AY507391	—	AY507520	AY507346	AY507474
Austrofossombronia australis (Mitt.) R. M. Schust.	New Zealand, *Stotler & Crandall-Stotler 4610*	AY507392	AY507434	AY507521	AY507347	AY507475
Austrofossombronia peruviana (Gottsche & Hampe) Stotler, Crand.-Stotl. & A. V. Freire	Venezuela, *Stotler & Crandall-Stotler 4187* (Ax)	AF536230	AY507435	AY507522	AY507348	AY507476
Blasia pusilla L.	U.S.A., North Carolina, *Renzaglia s.n.* (Ax)	AF536232	AY507436	AY507523	AY507349	AY507477
Calycularia crispula Mitt.	Japan, *Furuki s.n.*	AY507395	AY507437	AY507524	AY507350	AY507478
Cavicularia densa Steph.	Japan, *Nishimura s.n.*	AY507396	AY507438	AY507525	AY507351	AY507479
Diplophyllum obtusifolium (Hook.) Dumort.	U.S.A., Oregon, *Wheeler s.n.* [*ref. no. 351*] (Ax)	AY507397	AY507439	AY507526	AY507352	AY507480
Fossombronia angulosa (Dicks.) Raddi	Canary Isl., Teneriffe, *U. & E. Drehwald s.n.* [*ref. no. 371*] (Ax)	AY507398	AY507440	AY507527	AY507353	AY507481
Fossombronia foveolata Lindb.	U.S.A., Massachusetts, *D. & S. Williams s.n.* (Ax)	AY507399	AY507441	AY507528	AY507354	AY507482
Fossombronia sp. ined.	U.S.A., Arkansas, *Stotler & Crandall-Stotler 3940* (Ax)	AY507400	AY507442	AY507529	AY507355	AY507483
Frullania moniliata (Reinw., Blume & Nees) Mont.	Japan, *Mizutani s.n.* (Ax)	AY507401	—	AY507530	AY507356	AY507484
Haplomitrium blumii (Nees) R. M. Schust.	Malaya, *Furuki 384* (Ax)	AY507402	AY507443	AY507531	AY507357	AY507485
Haplomitrium mnioides (Lindb.) R. M. Schust.	Japan, *Furuki 7408* (Ax)	—	AY507444	AY507532	AY507358	AY507486
Hattorianthus erimonus (Steph.) R. M. Schust. & Inoue	Japan, *Furuki s.n.*	AY507403	AY507445	AY507533	AY507359	AY507487
Herbertus alpinus (Steph.) E. A. Hodgs.	New Zealand, *Stotler & Crandall-Stotler 4580*	AY507404	AY507446	AY507534	AY507360	AY507488
Hymenophyton flabellatum (Labill.) Dumort.	New Zealand, *Stotler & Crandall-Stotler 4511*	AY507406	AY507448	AY507535	AY507362	AY507489
Hymenophyton leptopodum (Hook. & Taylor) Steph.	New Zealand, *Stotler & Crandall-Stotler 4492*	AY507405	AY507447	AY507536	AY507361	AY507490
Isotachis multiceps (Lindenb. & Gottsche) Gottsche	Panama, *Stotler & Crandall-Stotler 3478* (Ax)	AY507407	AY507449	AY507537	AY507363	AY507491
Jensenia connivens (Colenso) Grolle	New Zealand, *Stotler & Crandall-Stotler 4535*	—	AY507450	—	—	—
Jubula hutchinsiae (Hook.) Dumort. ssp. *javanica* Steph.	Japan, *Kodama s.n.* (Ax)	AY507408	—	AY507538	AY507364	AY507492
Jungermannia leiantha Grolle	U.S.A., Illinois, *Stotler & Crandall-Stotler 107* (Ax)	AY507409	AY507451	AY507539	AY507365	AY507493
Lepicolea attenuata (Mitt.) Steph.	New Zealand, *Stotler & Crandall-Stotler 4586*	AY507410	AY507452	AY507540	AY507366	AY507494
Lobatiriccardia lobata (Schiffn.) Furuki	New Zealand, *Glenny & Kinser; det. Crandall-Stotler 4581*	AY507421	AY507462	AY507553	AY507378	AY507507
Marchantia polymorpha L.	[accessed from GenBank]	U87079	X04465	AF228786	NC001319	NC001319
Metzgeria conjugata Lindb.	U.S.A., Illinois, *Henson 1247* (Ax)	AY507411	AY507453	AY507541	AY507367	AY507495
Moerckia blytti (Moerch) Brockm.	U.S.A., Oregon, *Wagner s.n.*	AY507412	AY507454	AY507542	AY507368	AY507496
Moerckia flotoviana (Nees) Schiffn.	U.S.A., New York, *Kinser & Schuette 709*	AY507413	—	AY507543	AY507369	AY507497
Monoclea gottschei Lindb. ssp. *elongata* Gradst. & Mues	Puerto Rico, *Stotler & Crandall-Stotler 89-60* (Ax)	AY507414	AY507455	AY507544	AY507370	AY507498

Table 1. Continued.

Taxon	Voucher	GenBank accession no.				
		*rbc*L	*rps*4	*trn*L	*atp*B	*psb*A
Neohodgsonia mirabilis (Perss.) Perss.	New Zealand, *Stotler & Crandall-Stotler 4542*	AY507415	AY507456	AY507545	AY507371	AY507499
Noteroclada confluens Taylor ex Hook. & Wilson	Venezuela, *Freire & Crandall-Stotler 4189*	AF536228	—	AY507546	AY507372	AY507500
Pallavicinia lyellii (Hook.) Carruth.	U.S.A., Arkansas, *Marsh s.n.* (Ax)	AY507416	—	AY507547	AY507373	AY507501
Pellia epiphylla (L.) Corda	U.S.A., North Carolina, *Stotler & Crandall-Stotler 4414* (Ax)	U87085	AY507457	AY507548	—	AY507502
Petalophyllum ralfsii (Wilson) Nees & Gottsche ex Lehm.	U.K. J. Proskauer culture via D. Mueller-TAMU to ABSH (Ax)	AY507417	AY507458	AY507549	AY507374	AY507503
Phyllothallia nivicola E. A. Hodgs.	New Zealand, *Stotler & Crandall-Stotler 4537*	AY507418	AY507459	AY507550	AY507375	AY507504
Podomitrium phyllanthus (Hook.) Mitt.	New Zealand, *Stotler & Crandall-Stotler 4517*	AY507419	AY507460	AY507551	AY507376	AY507505
Porella navicularis (Lehm. & Lindenb.) Pfeiff.	U.S.A., California, *Stotler & Crandall-Stotler 3410* (Ax)	AY507420	AY507461	AY507552	AY507377	AY507506
Riccia huebeneriana Lindenb. ssp. *sullivantii* (Austin) R. M. Schust.	U.S.A., Illinois, *Stotler & Crandall-Stotler 92-175* (Ax)	AY507422	AY507463	AY507554	AY507379	AY507508
Scapania nemorea (L.) Grolle	U.S.A., Illinois, *Stotler & Crandall-Stotler s.n.* [*ref. no. 265*] (Ax)	AY507423	AY507464	AY507555	AY507380	AY507509
Schistochila appendiculata (Hook.) Dumort. ex Trevis.	New Zealand, *Stotler & Crandall-Stotler 4455*	AY507424	AY507465	AY507556	AY507381	AY507510
Sphaerocarpos texanus Austin	U.S.A., Illinois, *Stotler & Crandall-Stotler 1515* (Ax)	AY507425	AY507466	AY507557	AY507382	AY507511
Symphyogyna hymenophyllum (Hook.) Nees & Mont.	New Zealand, *Stotler & Crandall-Stotler 4452*	AY507426	AY507467	AY507558	AY507383	AY507512
Targionia hypophylla L.	U.S.A., California, *Stotler & Crandall-Stotler 3397* (Ax)	AY507427	—	—	AY507384	AY507514
Treubia lacunosa (Colenso) Prosk.	New Zealand, *Stotler & Crandall-Stotler 4561*	AY507428	AY507468	AY507559	AY507385	—
Treubia pygmaea R. M. Schust.	New Zealand, *Stotler & Crandall-Stotler 4582*	AY507429	AY507469	AY507560	AY507386	AY507515
Verdoornia succulenta R. M. Schust.	New Zealand, *Stotler & Crandall-Stotler 4602*	AY507430	AY507470	AY507561	AY507387	AY507516
Xenothallus vulcanicolus R. M. Schust.	New Zealand, *Stotler & Crandall-Stotler 4580*	AY507431	AY507471	AY507562	AY507388	AY507517
Outgroup Taxa						
Hornworts						
Anthoceros angustus Steph.	[accessed from GenBank]	AB087449	AB087443	—	D86545	AB087434
Anthoceros punctataus L.	[accessed from GenBank]	U87063	—	—	—	—
Megaceros aenigmaticus R. M. Schust.	[accessed from GenBank]	L13481	—	—	—	—
Nothothylas	[accessed from GenBank]	AF231888	—	—	—	—
Phaeoceros laevis (L.) Prosk.	[accessed from GenBank]	AF244562	—	—	—	—

Table 1. Continued.

Taxon	Voucher	GenBank accession no.				
		*rbc*L	*rps*4	*trn*L	*atp*B	*psb*A
Mosses						
Andreaea rothii F. Weber & D. Mohr	[accessed from GenBank]	AF231060	AF306952	AF478295	—	—
Andreaeobryum macrosporum Steere & B. M. Murray	[accessed from GenBank]	AF231059	—	—	—	—
Hypnum lindbergii Mitt.	[accessed from GenBank]	AB029390	AF143035	AF161128	—	—
Mnium hornum Hedw.	[accessed from GenBank]	AF226820	AF023796	AF231177	—	—
Polytrichum commune Hedw.	[accessed from GenBank]	U87087	AF231895	AF231907	—	—
Sphagnum cuspidatum Ehrh. ex Hoffm.	[accessed from GenBank]	AF231886	—	AF231903	—	—
Takakia lepidozioides S. Hatt. & Inoue	Canada, British Columbia, *Schofield 3176,* for *psb*A; others accessed from GenBank	AF244565	AF306950	—	—	AY507513

tution (Yang, 1994), plus a gamma distribution of rate variation among sites and a proportion of invariant sites [GTR + I + G, Lset Base = (0.3123 0.1627 0.1715) Nst = 6 Rmat = (1.5162 8.3773 0.7704 1.2306 10.1048) Rates = gamma Shape = 0.8047 Pinvar = 0.3690]. No bootstrap support was calculated for this matrix due to time constraints. MrBayes 2.01 (Huelsenbeck, 2000) was used to generate posterior probabilities for clades (lset basefreq = estimate nst = 6 rates = invgamma shape = estimate); two independent runs were conducted. The burnin for each run was determined by visualizing the curve of likelihood versus generations in Microsoft Excel, and a majority rule consensus of the remaining trees computed using PAUP* (Swofford, 2002). For the data sets excluding *Haplomitrium* and then *Treubia*, the 18 MPTs from the parsimony analysis were used as starting trees, with the excluded taxa pruned off, and swapped with TBR. No posterior probabilities were generated for these two partial data sets due to time constraints.

Results

The five chloroplast regions had a total aligned length of 4420 base pairs, of which 1842 were parsimony informative (Table 3). The proportion of parsimony informative sites in the included regions of sequence alignment ranged from 31.8% (*psb*A) to 61.4% (*rps*4). The value for *trn*L (57.9%) is artificially low, as a large proportion of the total alignment for this region was excluded due to ambiguities. Mean base frequencies for the five regions were biased towards A and T (Table 4).

All five regions produced significantly left-skewed g_1 values, indicative of phylogenetic structure to the data sets. Analyses for the five genes individually produced from two (*psb*A) to 324 (*trn*L) equally parsimonious trees (Table 5), with ensemble consistency indices (CI), excluding autapomorphies, ranging from 0.254 (*rbc*L) to 0.401 (*trn*L) and ensemble retention indices ranging from 0.526 (*atp*B) to 0.626 (*trn*L). Of the individual analyses, *rbc*L produced the most resolved strict consensus tree, with 53 nodes resolved. The MP analysis of the combined data set, however, had 17 more nodes with over 50% bootstrap (BS) support than the *rbc*L tree. MP analysis of the combined data found a total of 18 MPTs of length 11,834 and a consistency index (excluding uninformative characters) of 0.275 (Figs. 1, 2). These trees were distributed in three islands of 4, 4, and 10 trees, respectively. Forty-two nodes were resolved in the strict consensus tree (Fig. 1), and there were 42 nodes with over 50% BS support (Table 5).

Table 2. Primers used in PCR and sequencing reactions.

Gene/ region	Primer	Direction	Sequence	Reference
*atp*B	1163F	forward	5′-ATG GCA GAA TRT TTC CGA GAT RTY A	Wolf (http://bioweb.usu.edu/wolf/atpB%20primer%20map.htm)
*atp*B	672F	forward	5′-TTG ATA CGG GAG CYC CTC TWA GTG T	Wolf (http://bioweb.usu.edu/wolf/atpB%20primer%20map.htm)
*atp*B	LVWT 3F	forward	5′-TGG ATT GTR GGY GAG ARG ARC ATT A	Forrest (designed for this study)
*atp*B	384R	reverse	5′-GAA TTC CAA ACT ATT CGA TTA GG	Wolf (http://bioweb.usu.edu/wolf/atpB%20primer%20map.htm)
*atp*B	910R	reverse	5′-TTC CTG YAR AGA NCC CAT TTC TGT	Wolf (http://bioweb.usu.edu/wolf/atpB%20primer%20map.htm)
*atp*B	LVWT 3R	reverse	5′-TCY TCR CCY ACA ATC CAR GGT TG	Forrest (designed for this study)
*psb*A	501F	forward	5′-TTT CTC AGA CGG TAT GCC	C. Cox, Duke, NC (pers. comm.)
*psb*A	*trn*K2F	forward	5′-GAC GAG TTC CGG GTT CGA	C. Cox, Duke, NC (pers. comm.)
*psb*A	576R	reverse	5′-TGG AAT GGG TGC ATA AGG	C. Cox, Duke, NC (pers. comm.)
*psb*A	*trn*HR	reverse	5′-GAA CGA CGG GAA TTG AAC	C. Cox, Duke, NC (pers. comm.)
*rbc*L	M007	forward	5′-CCA CAA ACG GAG ACT AAA GC	Wheeler (pers. comm.), in La Farge et al. (2000)
*rbc*L	M636	forward	5′-GCG TTG GAG AGA TCG TTT CT	Lewis et al. (1997)
*rbc*L	M985	forward	5′-GGT ACT GTW GTA GGT AAA C	Lewis et al. (1997)
*rbc*L	NM34	forward	5′-GTT GTT GGA TTT AAA GCT GGT GTT	Wheeler (pers. comm.), in Cox et al. (2000)
*rbc*L	M745	reverse	5′-CTT CAC AWG TAC CTG CRG TAG C	Lewis et al. (1997)
*rbc*L	MtmRR	reverse	5′-GCT CTA ATC CAC TGA GCT AC	Wheeler (pers. comm.), in Cox et al. (2000)
*rps*4	*rps*5	forward	5′-ATG TCC CGT TAT CGA GGA CCT	Nadot et al. (1994)
*rps*4	trnAS	reverse	5′-TAC CGA GGG TTC GAA TC	Baker (pers. comm.), in Cox et al. (2000)
*trn*L	trnL/trnF-C	forward	5′-CGA AAT CGG TAG ACG CTA CG	Taberlet et al. (1991)
*trn*L	trnL/trnF-F	reverse	5′-ATT TGA ACT GGT GAC ACG AG	Taberlet et al. (1991)
*trn*L	trnL FOSS	reverse	5′-TCA CGG TCA AGC CAA CGG AT	Forrest (designed for this study)
*trn*L	trnL LWT	reverse	5′-GGR TAG AGG GAC TTG AAC	Forrest (designed for this study)

Table 3. Distribution of characters by locus.

Data set	Locus	Number of taxa	Length of locus included	Constant characters	Parsimony uninformative characters	Parsimony informative characters	% informative characters	g_1
All taxa	*rbc*L	59	1362	657	146	559	41.0	−0.4752
All taxa	*rps*4	48	581	145	79	357	61.4	−0.5555
All taxa	*trn*L	47	209	51	37	121	57.9	−0.6738
All taxa	*atp*B	48	1124	610	72	442	39.3	−0.5283
All taxa	*psb*A	47	1144	693	87	364	31.8	−0.4703
All taxa	combined	60	4420	2157	421	1842	41.7	−0.5789
Excluding *Haplomitrium*	combined	58	4420	2184	447	1789	40.5	−0.5531
Excluding *Treubia*	combined	58	4420	2172	423	1825	41.3	0.5751

ML analysis for the combined data set, including all taxa, recovered a single most likely tree (Fig. 3). Forty-six of the 58 nodes had posterior probabilities (PPs) of over 96%. Most of the BS support and PPs are for terminal clades (Figs. 1, 3), leaving the backbone of the tree lacking significant support.

The Metzgeriidae, or simple thalloid liverworts, as defined by Crandall-Stotler and Stotler (2000), are resolved as paraphyletic in all treatments. Both the complex thalloids and the leafy liverworts nest within the Metzgeriidae. The leafy liverworts are resolved as sister to the Metzgeriineae (*Metzgeria* Raddi, *Lobatiriccardia* (Mizut. & S. Hatt.) Furuki, *Verdoornia* R. M. Schust., and *Aneura* Dumort.), with 100% PP. The complex thalloid clade is sister to *Blasia* L. (100% PP, 66% BS). In the ML analysis, and in 4 of the 18 MPTs, a clade containing *Haplomitrium* and *Treubia* resolves as sister to all other liverworts (Figs. 2, 3). However, this clade has only 91% PP.

The maximum parsimony analysis with *Haplomitrium* deleted produced a more resolved strict consensus, with *Treubia* resolved as sister to the rest of the liverworts (78% BS). With *Treubia* deleted, MP analysis found only one MPT, in which *Haplomitrium* was nested within the Metzgeriales, as sister to *Pellia* Raddi. There is no significant BS support for this relationship. For both the minus *Haplomitrium* and the minus *Treubia* data sets, ML analyses produced topologies that were very similar to the respective MP analyses. Nothing in the ML analyses contradicted any supported clades in the MP analyses. *Treubia* resolved as sister to all other liverworts (Fig. 4), while *Haplomitrium* resolved as sister to *Calycularia* Mitt. (Fig. 5). The only topological differences between these two analyses are in the outgroup, with the mosses paraphyletic in the minus *Haplomitrium* analysis. This rooting problem probably relates to shared homoplasy between *Treubia* and the mosses, and should be correctable with better outgroup sampling.

Table 4. Mean base frequencies for five chloroplast loci and for the combined data sets for all taxa, for *Haplomitrium mnioides* only, and for *Treubia lacunosa* only.

Data set	Locus	A	C	G	T
All taxa	*rbc*L	0.282	0.185	0.235	0.299
All taxa	*rps*4	0.361	0.153	0.179	0.308
All taxa	*trn*L	0.321	0.194	0.228	0.256
All taxa	*psb*A	0.237	0.209	0.207	0.347
All taxa	*atp*B	0.307	0.179	0.225	0.289
All taxa	combined	0.289	0.185	0.219	0.307
H. mnioides	combined	0.270	0.214	0.235	0.281
T. lacunosa	combined	0.330	0.148	0.195	0.327

Discussion

The liverworts are one of the oldest, if not the oldest, groups of extant land plants (see Goffinet, 2000, for a detailed discussion of the different reconstructions of extant land plant lineages). The oldest accepted liverwort fossil, which is also the oldest fossil bryophyte, is *Pallaviciniites devonicus* (Hueber) R. M. Schust., a compression fossil from the Upper Devonian (Hueber, 1961). This plant is strikingly similar to frondose members of the Pallaviciniaceae Mig., such as *Jensenia* Lindb. and some species of *Pallavicinia* Gray (Krassilov & Schuster, 1984; Stewart & Rothwell, 1993), which resolve deep within the liverworts in our phylogenetic hypotheses. The minimal age of the fossil, and thus the group, is estimated at 375 m.y. (Krassilov & Schuster, 1984), and it is not unlikely, given that

Table 5. Results of parsimony analyses by locus.

Data set	Locus	Number of MP trees	Tree length	CI excluding uninformative characters	RI	Number of nodes in strict consensus tree	Number of nodes with >50% BS
All taxa	*rbc*L	4	3940	0.254	0.528	53	25
All taxa	*rps*4	216	2210	0.317	0.593	35	24
All taxa	*trn*L	324	536	0.401	0.626	32	16
All taxa	*atp*B	18	2898	0.275	0.526	30	27
All taxa	*psb*A	2	1887	0.281	0.561	42	23
All taxa	combined	18	11834	0.275	0.537	42	42
Excluding *Haplomitrium*	combined	8	11246	0.281	0.539	52	36
Excluding *Treubia*	combined	1	11537	0.278	0.536	54 (100%)	35

the major lineages of liverworts are basal to *Pallavicinia* in our reconstructions, that the date of origin of liverworts should be pushed further back considerably. It is important to bear in mind that this is the scale of time through which these lineages have been diversifying. Over such ages the possibility of superimposed substitutions must be considered, which leads to questions about the suitability of parsimony as a model for phylogenetic reconstruction. Maximum likelihood models of sequence evolution that account for multiple hits may well be more appropriate. In our analyses, the major supported clades are the same in both methods. However, for the placement of equivocal taxa, we feel that the maximum likelihood phylogram provides a better estimator.

The simple thalloid liverworts, or Metzgeriidae, are clearly a paraphyletic assemblage, while the leafy liverworts and the complex thalloids are both monophyletic. Upon reflection, this is not a surprising result. Both monophyletic groups here are defined at least partially on the basis of autapomorphies. For example, air chambers, archegonial involucres, and unistratose capsule walls are diagnostic of most complex thalloids, and leaves that develop from two superficial initials and acrogynous perichaetia are restricted to the leafy liverworts. In contrast, the simple thalloids are a heterogeneous assemblage of taxa that are largely defined by absences of these characters.

If one considers only those lineages that have significant posterior probabilities ($\geq$ 95%), five main clades of liverworts can be identified (Fig. 3). These correspond roughly to the following: (1) the suborder Pallaviciniineae of the Metzgeriales, designated here as Metzgeriales II; (2) the Fossombroniales Schljakov emend. Stotler & Crand.-Stotl.; (3) the Jungermanniidae Engl. emend. Stotler & Crand.-Stotl. or leafy liverworts; (4) the suborder Metzgeriineae of the Metzgeriales, designated here as Metzgeriales I; and (5) the Marchantiopsida or complex thalloids and *Blasia*. Two other well-supported lineages are the genera *Treubia*, which together with *Apotreubia* S. Hatt. & Mizut. comprises about 10 species (Stech et al., 2002), and *Haplomitrium*, comprising seven species (Bartholomew-Began, 1991). The positions of *Calycularia crispula* Mitt., *Pellia epiphylla* (L.) Corda, *Phyllothallia nivicola* E. A. Hodgs., and *Cavicularia densa* Steph. are unresolved in the strict MP consensus tree (Fig. 1) and are problematic in the ML tree (Fig. 3).

Different suites of morphological characters are associated with each of the major clades, providing some insight into possible phylogenetic trends in the morphological diversification of the Metzgeriidae. The clade designated as Metzgeriales II (Fig. 3) includes approximately 150 species, comprising the following genera: *Symphyogyna* Nees & Mont., *Xenothallus* R. M. Schust., *Jensenia*, *Pallavicinia*, *Podomitrium* Mitt., *Hattorianthus* R. M. Schust., *Moerckia* Gottsche, and *Hymenophyton* Dumort. The strikingly distinct *Phyllothallia nivicola*, which was segregated into its own remote suborder by Schuster (1968, 1992) and was later aligned with *Treubia* in the Treubiales Schljakov emend. Stotler & Crand.-Stotl. by Crandall-Stotler and Stotler (2000), is the sister taxon to this clade with 98% PP, but less than 50% BS support. Although this alignment of *Phyllothallia* E. A. Hodgs. with the Pallaviciniineae is at first glance surprising, this taxon actually does share some of the morphological characters that are diagnostic of the clade. These include antheridia and archegonia that are clustered on the dorsal midrib, associated with perigonial or perichaetial scales, respectively. The sporophyte is enclosed in a fleshy perigynium, a character shared with *Symphyogyna*, the gametophyte possesses a cuneate apical cell (Renzaglia, 1982),

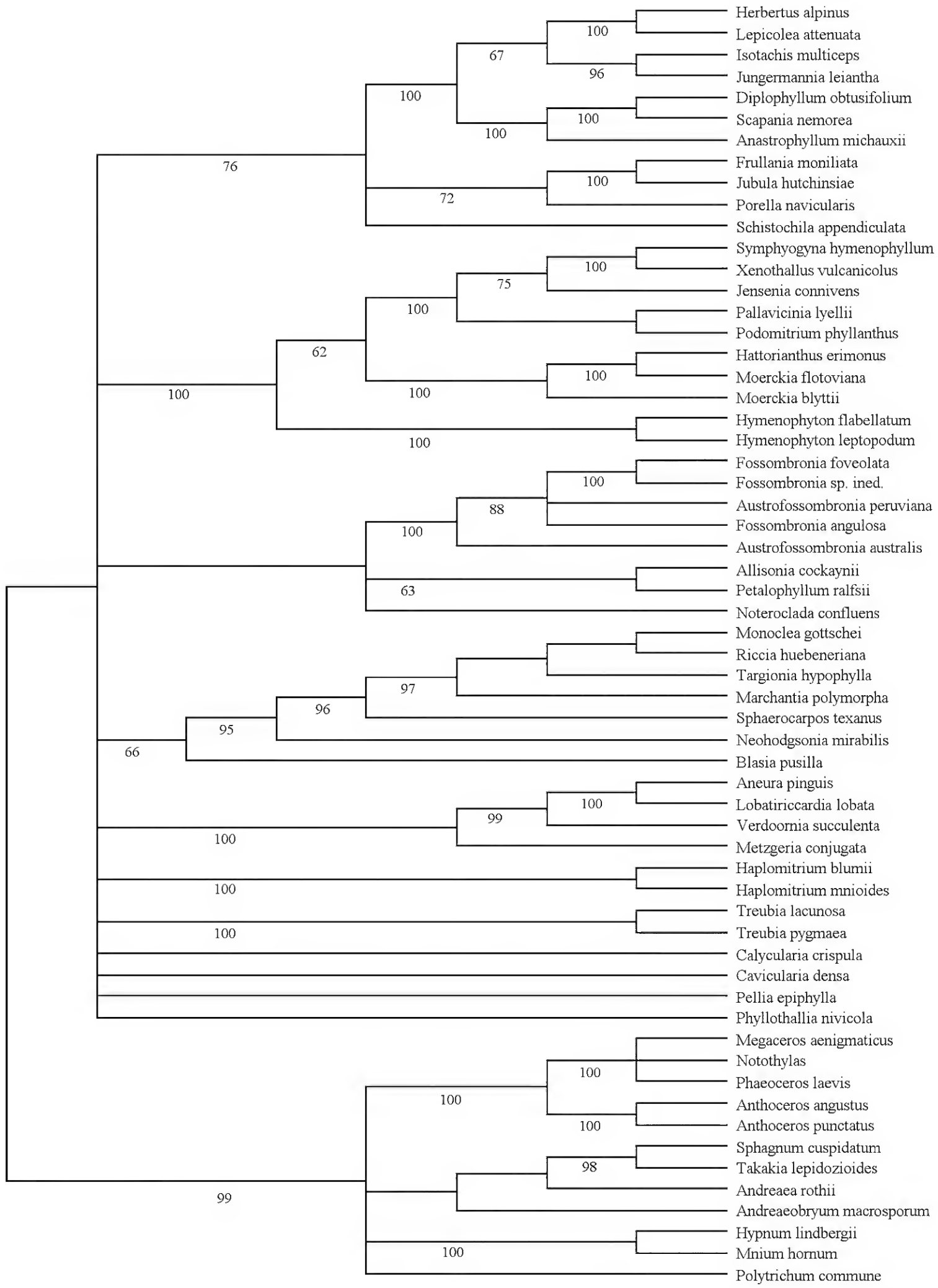

Figure 1. Strict consensus of 18 minimum length trees generated in a maximum parsimony analysis of *rbc*L, *rps*4, *trn*L, *atp*B, and *psb*A data, employing an heuristic search. The number of parsimony-informative characters = 1842; tree length = 11,834; CI = 0.275 excluding uninformative characters; RI = 0.537. Numbers below the branches are BS values above 50%.

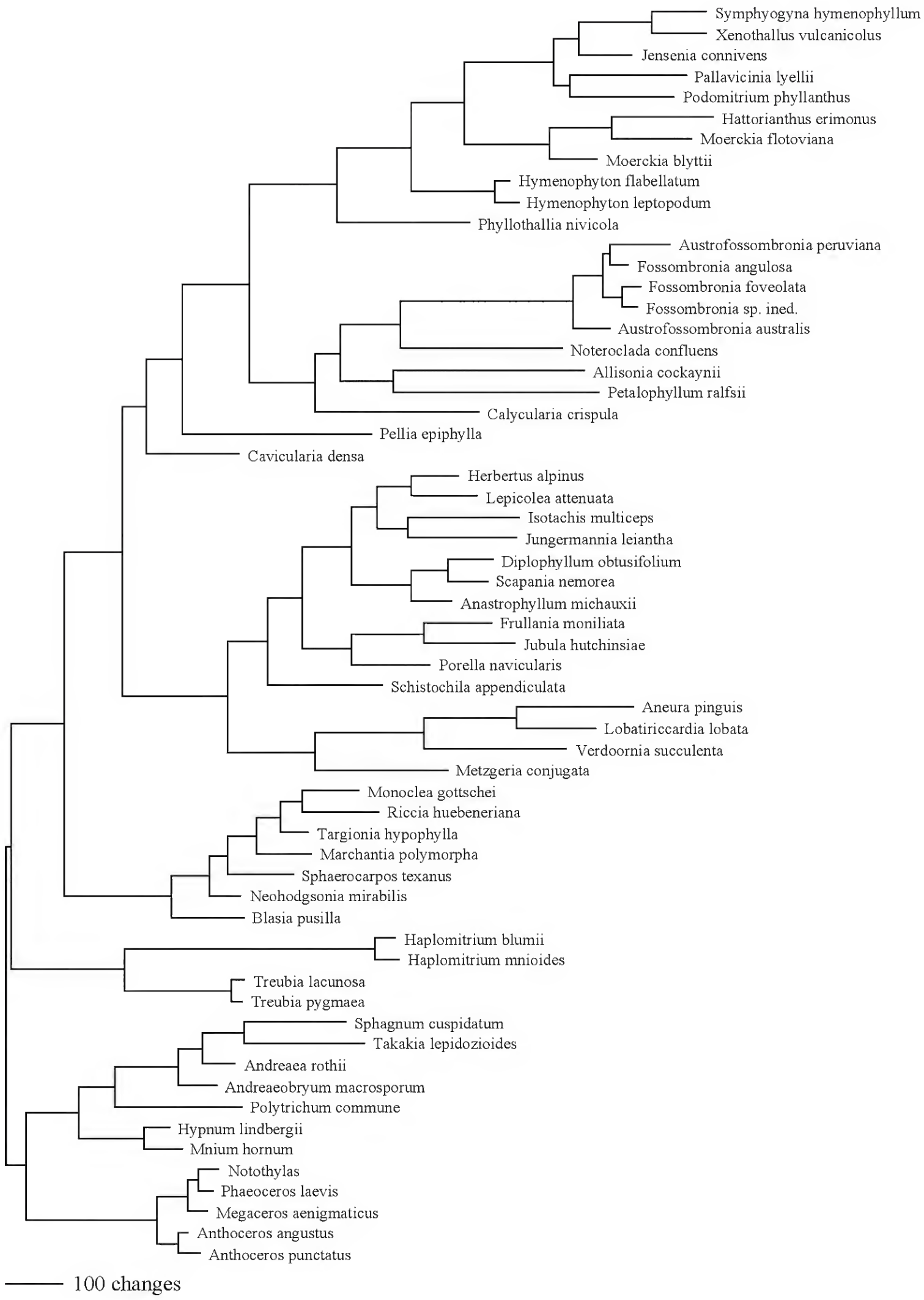

Figure 2. One of the 18 minimum length trees generated in a maximum parsimony analysis of *rbc*L, *rps*4, *trn*L, *atp*B, and *psb*A data, employing an heuristic search. The number of parsimony-informative characters = 1842; tree length = 11,834; CI = 0.275 excluding uninformative characters; RI = 0.537.

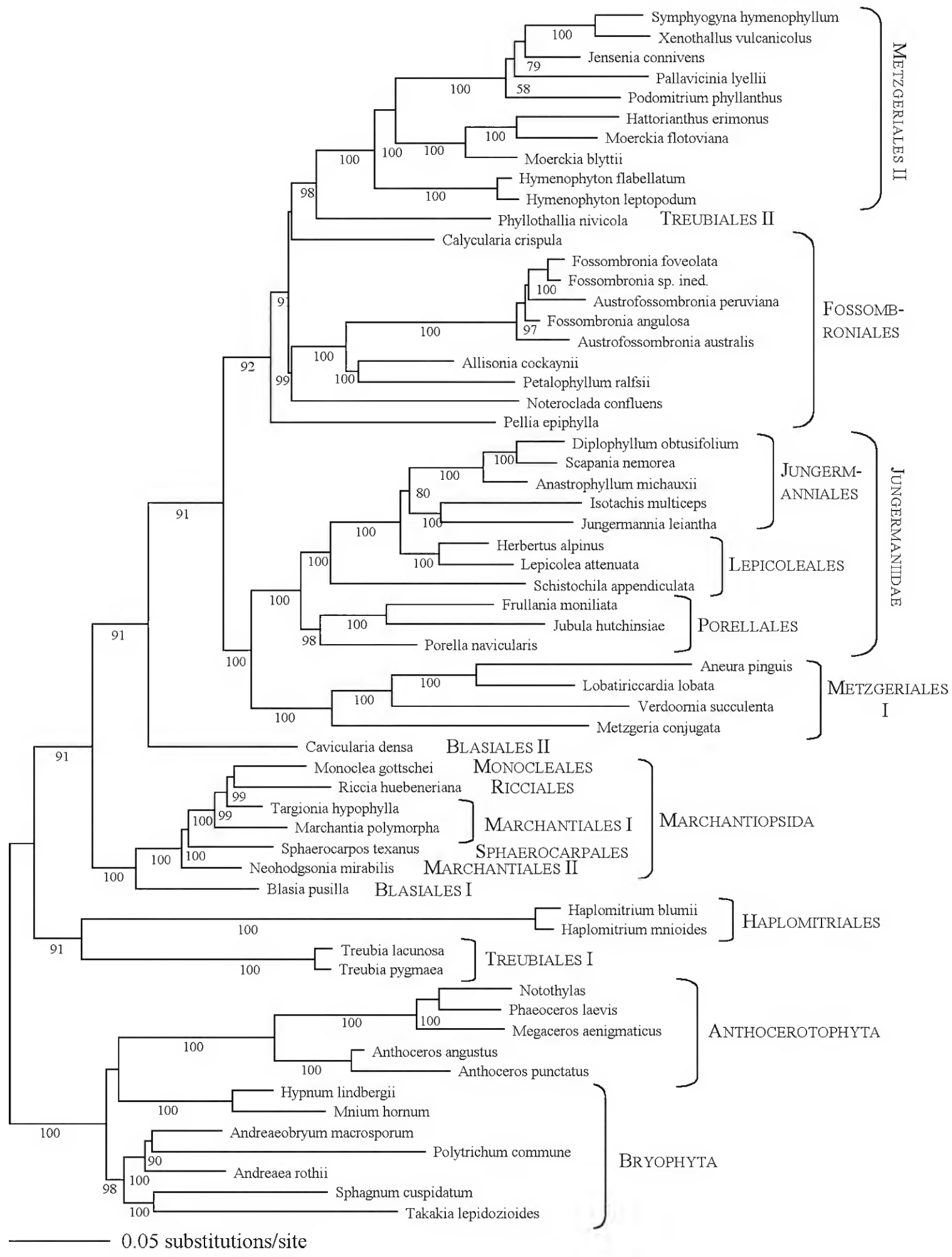

Figure 3. Likelihood analysis of *rbc*L, *rps*4, *trn*L, *atp*B, and *psb*A data. Single tree produced from an heuristic search (starting trees from MP analysis, TBR swapping) using the GTR substitution rate matrix with base frequencies A = 0.312, C = 0.163, G = 0.172, T = 0.353, plus a gamma distribution of rate variation among sites (approximated with alpha = 0.8045 and 4 categories) and a proportion of invariant sites (0.3690). Numbers below the lines, or to the right of nodes, are posterior probabilities estimated using MrBayes (lset basefreq = estimate nst = 6 rates = invgamma shape = estimate). −Ln likelihood = 57,286.93837. Classification at figure right taken from Crandall-Stotler and Stotler (2000).

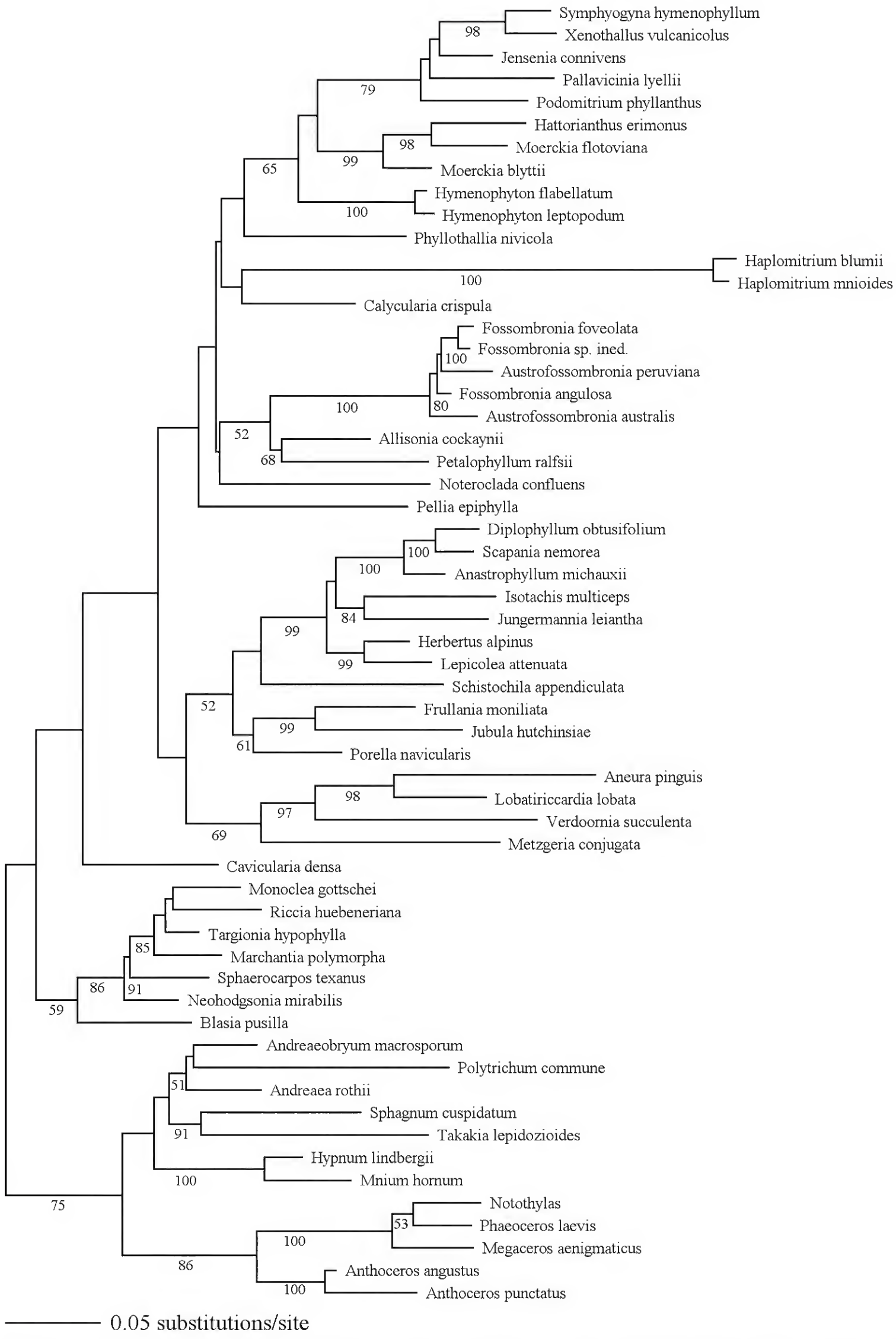

Figure 4. Likelihood analysis of *rbc*L, *rps*4, *trn*L, *atp*B, and *psb*A data, with *Treubia* excluded. Single tree produced from an heuristic search (starting trees from MP analysis, TBR swapping) using the GTR substitution rate matrix with base frequencies A = 0.312, C = 0.163, G = 0.172, T = 0.353, plus a gamma distribution of rate variation among sites (approximated with alpha = 0.8045 and 4 categories) and a proportion of invariant sites (0.3690). Numbers below the branches are BS values calculated under MP. −Ln likelihood = 55,986.65827.

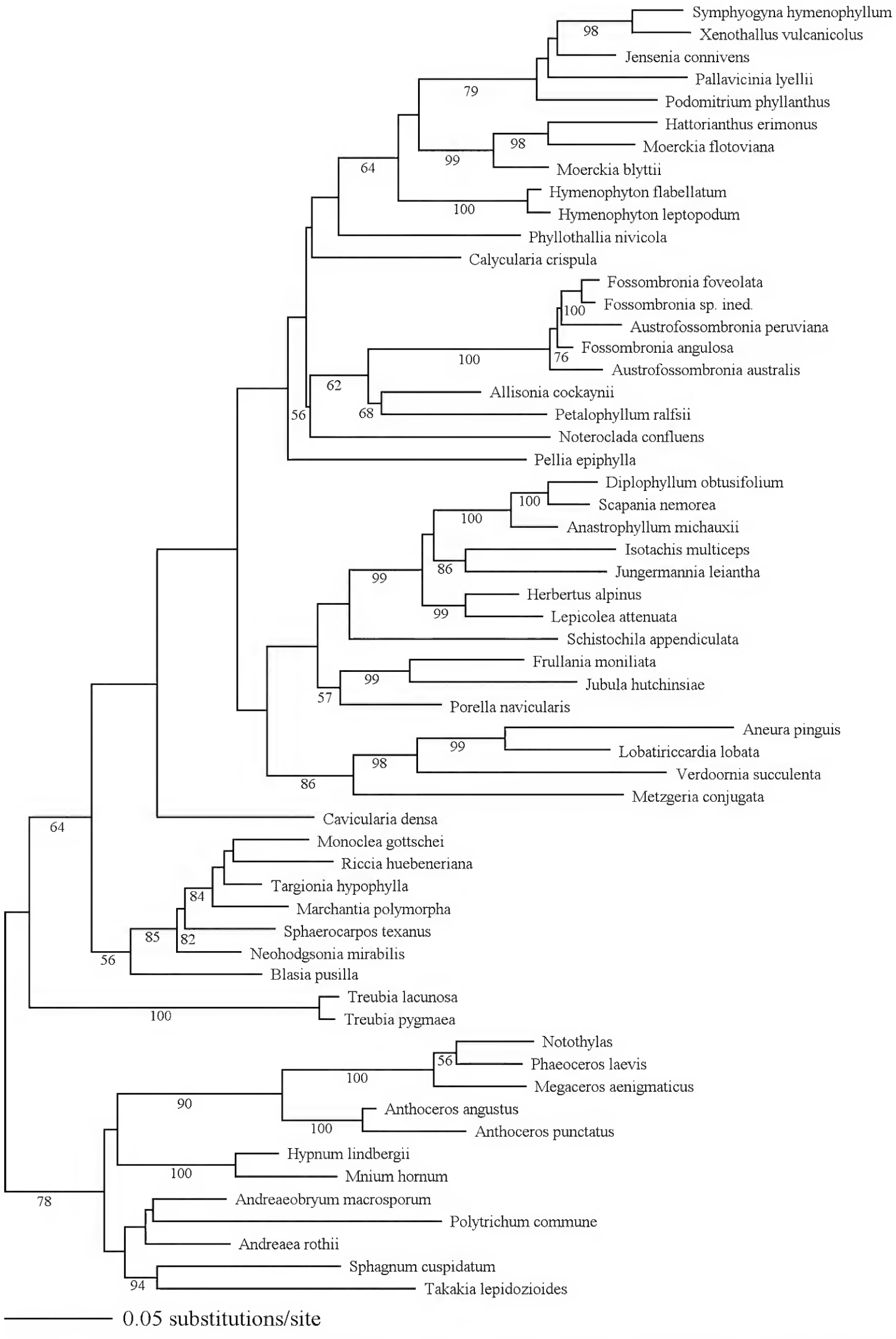

Figure 5. Likelihood analysis of *rbc*L, *rps*4, *trn*L, *atp*B, and *psb*A data, with *Haplomitrium* excluded. Single tree produced from an heuristic search (starting trees from MP analysis, TBR swapping) using the GTR substitution rate matrix with base frequencies A = 0.312, C = 0.163, G = 0.172, T = 0.353, plus a gamma distribution of rate variation among sites (approximated with alpha = 0.8045 and 4 categories) and a proportion of invariant sites (0.3690). Numbers below the branches are BS values calculated under MP. −Ln likelihood = 55,243.81851.

as do also *Moerckia* and *Symphyogyna,* and the capsule wall is 2-stratose as is common in the Pallaviciniaceae (Schuster, 1968). Differentiation of the gametophyte thallus into opposite leaf-like wings and long internodes is somewhat reminiscent of the lobate thalli of some species of *Symphyogyna.* Stalked slime papillae, which cover the broad apex on both dorsal and ventral surfaces, are also typical of most elements of the Pallaviciniaceae (Crandall-Stotler & Millar, 2003). Capsules have been described as spheroidal in *Phyllothallia* (Schuster, 1968), in contrast to the always elongate capsules of the Pallaviciniineae. However, this description was based on capsules at the spore-tetrad stage of development; mature capsules are undescribed for *Phyllothallia.* Kinser (2003) has shown that in *Moerckia,* capsules at this same stage are more or less spheroidal and that the mature ellipsoidal shape is due to elongation of wall cells during spore maturation. It is quite possible that mature capsules of *Phyllothallia* might also be elongate.

The sister group relationship of *Hymenophyton* with the genera of the Pallaviciniaceae is congruent with its placement in the Pallaviciniineae by Crandall-Stotler and Stotler (2000), rather than its status as a separate suborder with affinities to the Metzgeriineae as proposed by Schuster (1984, 1992). All taxa within the clade possess a thalloid growth form, with a furcated, dendroid habit characterizing *Hymenophyton, Jensenia,* and some species of *Pallavicinia* and *Symphyogyna.* In most genera, the thallus arises from a differentiated rhizome or stipe. Most taxa within the clade are vascularized; i.e., they possess hydrolyzed "conducting" strands in their thallus midribs. As documented by various authors, *Hymenophyton, Podomitrium, Pallavicinia, Jensenia,* and *Xenothallus* possess single, centrally positioned strands that are comprised of elongate, hydrolyzed cells, bearing thick walls and large plasmodesmata-derived pits (Hébant, 1977, 1980; Frey et. al., 1996; Ligrone & Duckett, 1996; Kobiyama, 2003). *Symphyogyna* also has these pallavicinioid-type strands, but often produces multiple strands per thallus. The *Moerckia/Hattorianthus* clade is characterized by thalli with two strands that are laterally displaced, except for *M. blyttii* (Moerch) Brockm., which lacks strands. In both *M. flotoviana* (Nees) Schiffn. and *Hattorianthus,* the cells comprising the strands lack plasmodesmata-derived pits in their walls (Hébant, 1977; Kobiyama, 2003). Wall structure is, however, very different in the two. In *M. flotoviana* the lateral walls are unthickened and appear to be partially hydrolyzed (Hébant, 1973, 1977: fig. 211), while in *Hattorianthus* the walls are thickened with an array of radially aligned, interwoven fibers (Kobiyama, 2003: fig. 136). In addition, the strand cells of *Hattorianthus* are much longer and narrower than surrounding parenchyma cells, in comparison to the strand cells of *M. flotoviana,* which are barely distinguishable from parenchyma cells in dimensions (Kinser, 2003). Structurally and developmentally, the strand cells of *Hattorianthus* are more highly differentiated than those of *Moerckia.* Since pallavicinioid-type strands occur in *Hymenophyton* (Hébant, 1980), both the *Moerckia* and the *Hattorianthus*-type strands are perhaps best interpreted as derived from the pallavicinioid type by developmental arrest, or paedogenesis. Regardless, although conducting tissues of other types are found outside the Metzgeriales II clade, the evolution of thick-walled, pallavicinioid-type strands occurred only in this clade.

Within the clade, *Xenothallus,* a rare New Zealand endemic, is resolved as a sister taxon to *Symphyogyna,* with 100% PP and BS support, a relationship that is also supported by new data that demonstrate the anatomical similarities of their sporophytes (Crandall-Stotler, 2003). *Jensenia* is resolved as distinct from *Pallavicinia,* but even though this result is concordant with the morphological evidence of Schuette (2003), it must be considered provisional since the data set for *Jensenia* is incomplete. *Hattorianthus,* which Schuster (1992) aligned with *Pallavicinia,* is nested within *Moerckia* as sister to *M. flotoviana,* also with 100% PP and BS support. Morphological studies of Kinser (2003) are in accord with this result and support the transfer of *Hattorianthus* from the subfamily Pallavicinioideae Migula into the subfamily Moerckioideae R. M. Schust. In fact, the only character that distinguishes these two genera is strand cell anatomy (Kinser 2003).

The placement of *Calycularia* has traditionally been problematic. Named as a monotypic taxon by Mitten (1861), the genus was originally aligned with *Blyttia* Endl. [= *Pallavicinia*] and *Moerckia.* Evans (1939) included *C. crispula* with *Pellia* and *Noteroclada* Taylor ex. Hook. & Wilson in the Pelliaceae H. Klinggr., while most recently this taxon has been aligned with *Allisonia* Herzog in the Allisoniaceae (R. M. Schust. ex Grolle) Schljakov (Schuster & Inoue, 1975; Crandall-Stotler & Stotler, 2000). Unfortunately, it is not possible to be certain of the relationships of this taxon from these analyses; ML puts it sister to the *Phyllothallia*/Pallaviciniineae clade (Fig. 3), and MP puts it sister to the Fossombroniales clade (Fig. 2). Its placement lacks support from either methodology. The most that can be

said with any confidence is that it does not belong in any of our well-supported clades.

The clade designated as the Fossombroniales (Fig. 3) comprises the genera included by Crandall-Stotler and Stotler (2000) in the Fossombroniales, except for *Calycularia* and *Pellia*. Many of the taxa in this clade have a leafy or nodal thallus organization. Only *Petalophyllum* Nees & Gottsche ex Lehm. and *Allisonia*, which resolve as sister to the genera of the Fossombroniaceae Hazsl. in the ML analysis (100% PP), are thalloid, although the thallus of *Petalophyllum* forms large, conspicuous lamellae that have been likened to leaves (Schuster, 1992). In all genera except *Allisonia* and *Noteroclada*, two rows of small, usually toothed, or divided, scales occur near the apex on the ventral surface of the midrib (J. Long & Crandall-Stotler, 2003). In *Allisonia* these are replaced by uniseriate ventral slime hairs, while in *Noteroclada* there are elongate, stalked slime papillae on the ventral surface of the midrib. Both antheridia and archegonia are scattered and typically unprotected, or naked, in the "leafy" forms, but are clustered and associated with scales in the thalloid *Allisonia* and *Petalophyllum*. The most consistent character of the clade is the spheroidal shape of the capsule, which becomes only slightly elongate in some species of *Austrofossombronia* R. M. Schust. In addition, capsule dehiscence is irregular, or non-valvate in all taxa but *Noteroclada*. Based on capsule shape, *Pellia* would be expected to resolve within this clade. Further, *Pellia* and *Noteroclada* share precocious, endosporic germination (Schuster, 1992). However, the position of *Pellia*, much like *Calycularia*, has not been resolved with any support in our analyses. The Fossombroniaceae themselves are strongly supported as monophyletic (100% PP and BS), although the monophyly of *Austrofossombronia* and *Fossombronia* are not supported. Increased sampling from within this family using sequences from *rps*4, *trn*L, and *rbc*L supports the non-monophyly of both these genera (Forrest et al., 2003).

The subclass Jungermanniidae, or true leafy liverworts, form a third, well-supported clade in our analyses, with 100% PP and 76% BS support. The few jungermannioid taxa that we have included in our analyses are resolved into two main clades, one of which comprises the Porellales (R. M. Schust.) Schljakov emend. Stotler & Crand.-Stotl. (98% PP, 72% BS) and the second, a paraphyletic assemblage of the Lepicoleales Stotler & Crand.-Stotl. and Jungermanniales H. Klinggr. emend. Stotler & Crand.-Stotl. (100% PP, 100% BS without *Schistochila* Dumort.). This topology is in agreement with more detailed analyses of the leafy liverworts (Davis, 2004 cf. this volume; He-Nygrén et al., 2004 cf. this volume) and supports the recognition of the Porellales (R. M. Schust.) Schljakov emend Stotler & Crand.-Stotl. as a distinctive, monophyletic group. It does not, however, support the recognition of this order as the most highly derived group of liverworts as suggested by modern systems of liverwort classification (Schuster, 1984; Crandall-Stotler & Stotler, 2000). Evolution within the leafy liverworts is addressed in more detail by both Davis (2004) and He-Nygrén et al. (2004) in this volume.

The leafy liverwort, or jungermannioid, clade is, itself, nested in the simple thalloids as a sister group to the clade designated as Metzgeriales I. This relationship is strongly supported in ML analyses with 100% PP (Fig. 3) but is unresolved in a MP strict consensus (Fig. 1). The Metzgeriales I clade is supported in both ML (100% PP) and MP (100% BS) analyses and comprises the genera of the Metzgeriineae and the New Zealand endemic *Verdoornia*. The nesting of *Verdoornia* between the Aneuraceae H. Klinggr. and the Metzgeriaceae H. Klinggr., which was also seen in the analyses of Stech and Frey (2001), is unexpected in light of Schuster's (1999: 73) description of it as "one of the most isolated Metzgeriales genera." Actually, however, this genus possesses many of the morphological features that are frequent in the clade. Gametophytes of plants in the Metzgeriales I clade vary from large, fleshy, undifferentiated thalli, like those of *Verdoornia* and *Aneura*, to small, delicate thalli that are clearly differentiated into a stem-like midrib and wing, as in *Metzgeria* Raddi. *Verdoornia*, like all other taxa, possesses an apical cell that is lenticular in geometry (Crandall-Stotler, unpublished). Thallus apices are protected ventrally by fairly long, stalked slime papillae in *Verdoornia* as well as the Aneuraceae, or by single-celled slime papillae in the Metzgeriaceae (Renzaglia, 1982). Reduced archegonia with very short to absent necks are diagnostic of the clade. Although Schuster (1999) describes the neckless archegonia of *Verdoornia* as unique, they are, in fact, exactly like the archegonia of the Aneuraceae and Metzgeriaceae. In *Verdoornia* the gynoecium is formed on the dorsal surface of the leading thallus, in the Aneuraceae it is formed on short, determinate lateral branches, and in the Metzgeriaceae it is formed on reduced, ventral branches that are endogenous in origin. In both *Verdoornia* and the Aneuraceae, the antheridia are sunken to varying degrees in pits and are surrounded either singly, or in groups of 2 or 3 (to 5), by flask-shaped to tubular outgrowths of the thallus (compare Renzaglia, 1982: fig. 695 with Schuster, 1999: fig. 1). In all taxa of the clade,

sporophytes are enclosed in fleshy tissue, either a shoot calyptra or a perigynium, and in the Aneuraceae and *Verdoornia*, capsules bear apical elaterophores. In addition to this unexpected alignment of *Verdoornia* with the Aneuraceae, the resolution of this clade as sister to the Jungermanniideae does not support traditional hypotheses that regard the suborder Metzgeriineae as the most derived group of the simple thalloid liverworts.

The Marchantiopsida, or complex thalloid liverworts, are reconstructed as monophyletic, with 100% PP and 95% BS support. This clade has a suite of morphological characters also supporting its monophyly, such as unistratose capsule walls and unlobed spore mother cells (Bischler, 1998). Further, mitochondrial RNA editing has been found in all major land plant groups except the Marchantiopsida (Steinhauser et al., 1999), and some mitochondrial introns are found in all groups except marchantioids (Qiu et al., 1998). The relationships of the marchantioid taxa included in our analyses largely mirror the topologies in Boisselier-Dubayle et al. (2002) and Wheeler (2000).

The hypothesis that the Monocleales R. M. Schust. represents a basal lineage within the complex thalloids has been put forward by Grolle (1983), Schuster (1984, 1992), and Grolle and Long (2000). However, we, like Lewis et al. (1997) and Boisselier-Dubayle et al. (2002), reconstruct *Monoclea* Hook. within the Marchantiidae Engl. Although *Monoclea* superficially appears to blend characters of marchantioid and jungermannioid lineages, a re-interpretation of its morphology by Crandall-Stotler and Stotler (2000) reconciles it with the complex thalloids. Wheeler (2000) suggested that the gametophyte phase of *Monoclea* has undergone reductive evolution from a more "typical" marchantioid ancestor, possibly due to an aquatic or semi-aquatic ancestry, while its massive sporophyte may reflect the retention of plesiomorphic states for the clade. Previous morphology-based analyses, as well as classifications not based on an explicit phylogeny, have also been confounded, Boisselier-Dubayle et al. (2002: 74) suggested, by covariation between "life history patterns and morpho-anatomical traits." When resources are limited on a periodic basis, taxa evolve a high growth rate and an early, high reproductive effort. These taxa are characterized by morphological reductions such as those of *Sphaerocarpos* Boehm. and *Riccia* L. On the other hand, taxa that occupy mesic habitats retain complex morphological traits, like antheridiophores and archegoniophores. Thus *Neohodgsonia* Perss., which we, like Boisselier-Dubayle et al. (2002: 75), resolve "at the base of the extant Marchantiopsida," i.e., as sister to all other complex thalloids, with support values of 100% PP and 95% BS, has elaborate gametangiophores, while taxa like *Monoclea*, which appear in the crown group, lack such structures.

The simple thalloid liverwort *Blasia* is reconstructed as sister to the complex thalloid clade with 100% PP and 66% BS support. Carothers (1973) showed that the blepharoplast of *Blasia* is most like that of complex thalloids, and Pass and Renzaglia (1995) recommended realigning *Blasia* with the marchantioids based on these similarities. Other features that *Blasia* shares with this clade include a small, cuneate apical cell and fairly large, persistent ventral scales that are arranged in two rows. It should be noted, however, that this latter character is also common in the Fossombroniales. As coded in Crandall-Stotler and Stotler (2000), the perichaetium or female involucre of *Blasia* is comparable to the marchantioid involucre as defined by Bischler (1998) and, in fact, is identical to that found in *Monoclea*. At the same time, however, the multistratose capsule wall and lobed spore mother cells (Leitgeb, 1874: pl. III, fig. 16) are diagnostic features of the Jungermanniopsida (Schuster, 1992).

Analyses of 5 cpDNA loci yield no support in terms of PP or BS for the positions of five genera, namely, *Calycularia*, *Pellia*, *Cavicularia* Steph., *Haplomitrium*, and *Treubia*. Morphological characters suggest an affinity between *Calycularia*, *Pellia*, and the Fossombronialean clade. *Cavicularia* has been traditionally segregated with *Blasia* in a single family, the Blasiaceae H. Klinggr. The two taxa differ from all other Metzgerialian liverworts, but resemble each other in having *Nostoc*-filled ventral auricles (Inoue, 1976; Schuster, 1992). In both taxa the archegonia and sporophyte are enclosed by thallus tissue, as is also the case in *Monoclea*. There are, however, significant differences between *Blasia* and *Cavicularia*, which include distinctive perigonia and the lack of stellate gemmae in *Cavicularia*. *Cavicularia* has a strictly thalloid organization and possesses three strands of hydrolyzed, *Moerckia*-like strand cells in its midrib (Kobiyama, 2003), whereas *Blasia* has a lobed to "leafy" thallus and appears to lack such strands. In separate MP analyses of the genes *psb*A (91% BS, not shown) and *trn*L (< 50% BS, not shown) *Cavicularia* was resolved as sister to *Blasia*, but separate analyses of *rbc*L, *rps*4, and *atp*B all gave results similar to those of the combined five-gene analysis. If our current reconstruction is correct, with *Blasia* sister to the complex thalloids and *Cavicularia* sister to most of the Jungermanniopsida, we could hypothesize that the *Blasia*/*Cavicularia* morphotype ("Blasi-

aceae") represents the basal condition for the liverworts, excepting *Haplomitrium* and *Treubia*, from which both the Marchantiopsida and the Jungermanniopsida diverged. Clearly, before we invest too much into this hypothesis, we need increased support measures for the branches at the base of our reconstruction.

Haplomitrium and *Treubia* are resolved as sister to the rest of the liverworts. Although there is good support for the monophyly of *Haplomitrium* and for that of *Treubia*, with 100% PP and BS for both, their sister group relationship lacks support (91% PP, < 50% BS). Both genera are isolated morphologically and genetically, i.e., both are on long branches, as can be seen in the MP and ML phylograms (Figs. 2, 3). Thus, it is possible that this clade is influenced by long branch attraction. The base compositions for *Treubia* are quite different from the mean values for the data set (Table 4), which can certainly confound parsimony-based methods. The lack of resolution in the strict consensus of the 18 MP trees (Fig. 1) is largely due to the position of *Haplomitrium*, which is sister to *Pellia* in some MPTs and to *Treubia* in others. Certainly this is a case where the addition of a morphological data set might aid resolution, as might also increased sampling within *Haplomitrium*. Analyses based on morphology alone have also produced equivocal results for these two genera. For example, Crandall-Stotler and Stotler (2000) resolved *Haplomitrium* as sister to a *Fossombronia*/leafy liverwort clade and *Treubia* as sister to *Pellia*. Garbary and Renzaglia (1998) suggested a relationship between *Haplomitrium* and *Monoclea*, with *Treubia* sister to a *Riccardia* Gray/*Pallavicinia*/leafy liverwort clade, but Garbary et al. (1993), using only spermatid characters, resolved a sister group relationship between *Treubia* and *Haplomitrium*. The only previously published molecular analysis to include these taxa is an MP analysis of partial sequences of the *trn*L intron (Stech & Frey, 2001). *Haplomitrium* is unresolved within a simple thalloid clade, and *Treubia* is resolved as sister to all the other liverworts, but without support. Qiu (2003), using one mitochondrial, three chloroplast, and two nuclear genes, also reconstructed a *Treubia*/*Haplomitrium* clade as sister to the rest of liverworts. In light of these additional studies, dismissal of this *Treubia*/*Haplomitrium* relationship as an artifact of homoplasy is premature. There are some morphological characters shared by these genera, including tetrahedral apical cells, stalked slime papillae, and a lack of perichaetial scales. Blepharoplast morphology also suggests a relationship between *Treubia* and *Haplomitrium* (Carothers & Rushing, 1990). Even back in 1898, Goebel (p. 266) suggested that the Calobryaceae Cavers [= Haplomitriaceae Dedecek] show an affinity with *Treubia*.

While the relationship between *Treubia* and *Haplomitrium* is at best tentative, we can be more certain of the position of *Treubia*. We have tested whether the phenomenon of branch attraction is responsible for the placements of *Treubia* and/or *Haplomitrium* by excluding first one and then the other from our analyses. Without *Treubia*, *Haplomitrium* resolves as sister to *Calycularia* in ML analysis (Fig. 4) or to *Pellia*, subtended by *Calycularia* in MP analysis. In both cases its clade is sister only to the Metzgeriales II. It never resolves as sister to all other liverworts. These results suggest that we may be facing some sort of long branch problem with the placement of *Haplomitrium* in parsimony and in likelihood analyses. However, without *Haplomitrium* included, *Treubia* still resolves as sister to all other liverworts, with 78% BS support (Fig. 5). This relationship is shown regardless of whether our reconstructions are parsimony- or likelihood-based, despite the skewed base composition found in *Treubia* (Table 4).

There are two extant genera in the Treubiaceae Verd. The status of *Apotreubia*, established by Hattori et al. (1966), has been questioned. It was reduced to *Treubia* by Schuster and Scott (1969), but resurrected by Schuster and Konstantinova (1995). Stech et al. (2002) are the only authors to have sequence data from this genus. In their partial *trn*L intron analysis, it resolves as sister to a clade comprising the three included species of *Treubia*. Given the pivotal placement of *Treubia* as sister to all the other liverworts, including *Apotreubia* in more comprehensive studies could have an important effect on the backbone of the liverwort phylogeny. The inclusion of this genus must surely be a goal for future studies.

The sizes of supported clades within the liverworts vary greatly, with only about 10 species each in the Haplomitriales and the Treubiales, ca. 80 in the Fossombroniales, ca. 150 in the Pallaviciniineae, ca. 250 in the Metzgeriineae, ca. 350 in the Marchantiopsida, and somewhere in the region of 6000 to 7000 in the Jungermanniidae (Crandall-Stotler & Stotler, 2000, unpublished). We can be reasonably confident that the species numbers in the simple thalloid clades will not alter much with the addition of missing taxa, since of the 14 unsampled genera, which include *Apotreubia*, *Sewardiella* Kashyap, *Sandeothallus* R. M. Schust., *Greeneothallus* Hassel, *Seppeltia* Grolle, *Symphyogynopsis* Grolle, *Makinoa* Miyake, *Cryptothallus*

Malmb., *Riccardia*, *Mizutania* Furuki & Z. Iwats., *Vandiemenia* Hewson, *Apometzgeria* Kuwah., *Austrometzgeria* Kuwah., and *Steereella* Kuwah., 13 are small or monotypic. Only *Riccardia* displays substantial species diversity, with as many as 100 species worldwide (Schuster, 1992). The incredible species diversification of the leafy liverworts is thought to be a radiation associated with the diversification of the angiosperms, which opened up a range of new habitats on angiosperm bark and leaves (Ahonen et al., 2003). While it will be of some interest to test where on the phylogeny the hypothesized speciation rate increase occurs, i.e., whether it also explains the relatively large number of species in *Riccardia* and *Metzgeria* or is strongly linked to some key innovation in the leafy liverwort clade, such comparisons must await a better supported backbone for the liverwort phylogeny and increased taxon sampling. The sister taxon to most of the liverwort species, whether it is *Treubia* alone or a *Treubia*/*Haplomitrium* clade, is comparatively species-poor, with only 10 to 20 species as opposed to almost 8000. This discrepancy in lineage diversifications, or lack of topological balance, appears to be a relatively common phenomenon in phylogenies (Pearson, 1999). Pearson (1999: 405) explains this pattern as "the result of the relative success of apomorphic taxa over their more pleisiomorphic sisters, where success is measured in terms of resistance to extinction or propensity for speciation."

From superficial scrutiny of the phylogram topologies from MP and ML analyses (Figs. 2, 3), a molecular evolutionary rate change is apparent between the complex thalloid/*Blasia* clade and the rest of the liverworts. This rate change, which was also observed by Lewis et al. (1997) for an *rbc*L data set, is apparent not only in our combined analysis, but also in our individual analyses of *rbc*L, *psb*A, *rps*4, and *atp*B. While herein is not the place to go into these results in any great detail, it does certainly appear that rates of nucleotide change in the chloroplast genome are lower in the complex thalloids than they are in the other liverworts. Beckert et al. (1999) also reported a difference in substitution rates for *nad*5, with slower sequence evolution in the complex than the simple thalloids. This ties in very strongly with our preliminary studies on this region (Forrest & Crandall-Stotler, unpublished). It will be informative to contrast the situation in plastid genes with the small subunit ribosomal RNA nuclear gene, where accelerated rates have been found in the complex thalloids (Capesius & Bopp, 1997; Forrest & Crandall-Stotler, unpublished).

Conclusions

Simple thalloid liverworts are a paraphyletic assemblage that encompass the complex thalloid and the leafy liverwort clades. *Treubia*, with or without *Haplomitrium*, is sister to all other liverworts. The Pallaviciniineae and the Fossombroniales both form well-supported clades, as do the Metzgeriales plus the Jungermanniidae, and *Blasia* plus the Marchantiopsida. The Jungermanniidae and Marchantiopsida are also resolved as well-supported monophyletic groups under our sampling strategy. It is premature to name clades—for example, the position of *Haplomitrium* is currently highly equivocal—although there are certainly some clades that likely will require nomenclatural changes. As an example, both the Metzgeriales and the Treubiales, as delimited in Crandall-Stotler and Stotler (2000), are polyphyletic, each comprising two well-supported clades. Future analyses will add data sets from the nuclear small and large subunit ribosomal RNA genes and the mitochondrial *nad*5 gene, as well as increase taxon sampling within the simple thalloid liverworts. These additional data should allow us to better resolve the backbone of our phylogeny and the placement of problematic taxa like *Haplomitrium* and *Cavicularia*. Taxonomic modifications should be postponed until such analyses are completed.

It is also premature to draw conclusions as to the evolutionary trends or patterns seen in morphological characters within the simple thalloids. At first glance, our phylogeny does not seem to support most of the assumptions of linear character transformations postulated by Schuster (1992: 340). Considering apical cell geometry, for example, tetrahedral apical cells do occur in both *Treubia* and *Haplomitrium*, which might suggest that this geometry is the plesiomorphic state, as Schuster (1992) proposed. But, sister taxa to major clades, like *Blasia*, *Cavicularia*, and *Pellia*, all possess cuneate apical cells, while other simple thalloid taxa with tetrahedral apical cells, namely, *Noteroclada* and *Petalophyllum*, are nested between taxa with cuneate apical cells and taxa with lenticular ones. In the well-supported leafy liverwort/Metzgeriineae clade, tetrahedral apical cells characterize all the leafy liverworts and lenticular apical cells, all members of the Metzgeriineae. In fact, patterns of character evolution may differ among the clades. In order to analyze such trends, future studies will incorporate a comprehensive data matrix of morphological characters in a total evidence analysis.

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PHYLOGENETIC RELATIONSHIPS BETWEEN LOPHOZIACEAE AND SCAPANIACEAE BASED ON CHLOROPLAST SEQUENCES[1]

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Abstract

Lophoziaceae and Scapaniaceae (Marchantiopsida, Jungermanniales) have traditionally been kept apart as separate families. Taxonomic and evolutionary studies on *Anastrophyllum* (Lophoziaceae) included a molecular analysis to evaluate relationships within *Anastrophyllum* and to study phylogenetic relationships to other members of the Lophoziaceae and other families. Two non-coding regions of the chloroplast genome were sequenced, the *trn*L intron and the intergenic region between *atp*B and *rbc*L, for 23 leafy liverworts. Phylogenetic trees based on the *trn*L matrix were unresolved. The *atp*B-*rbc*L analysis and the combined data analysis both generated a single most parsimonious tree. Although both chloroplast regions were evolving rapidly and hypervariable regions had to be excluded, phylogenetic relationships between taxa were sufficiently clarified. In the combined tree *Anastrophyllum* and Scapaniaceae taxa analyzed (5 spp.) appeared to be monophyletic groups and both were nested within the Lophoziaceae subfamily Lophozioideae. The nesting of Scapaniaceae within Lophoziaceae is particularly interesting, as it suggests that Lophoziaceae is paraphyletic and that Scapaniaceae has evolved from within the Lophoziaceae. However, it is considered premature to merge the two families into a single enlarged Scapaniaceae. *Jamesoniella* is sister to the Lophoziaceae/Scapaniaceae clade. *Delavayella* (Delavayellaceae) is unrelated to the Scapaniaceae. The relationship of Jungermanniaceae to Lophoziaceae remains unclear most likely due to the low sampling (3 spp.) of the Jungermanniaceae.

Key words: *Anastrophyllum*, *atp*B-*rbc*L intergenic region, chloroplast DNA, Delavayellaceae, Jungermanniales, leafy liverworts, Lophoziaceae, Scapaniaceae, *trn*L intron.

In recent years molecular phylogenetic studies using nucleotide sequencing on bryophytes have become numerous (Goffinet & Hax, 2001; Goffinet, 2003), but most of those including liverworts have tested hypotheses of evolution at relatively high levels, particularly in the context of evolution of land plants, and the ordinal relationships of liverworts, hornworts, and mosses, as summarized by Goffinet (2000). These studies have included only sparse sampling of Jungermanniales. Although a number of studies in mosses, such as *Sphagnum* (Shaw, 2000), have looked at lower level relationships among species, sections, subgenera, and related genera, very few such studies have taken place in liverworts; examples are those on species of *Asterella* (Marchantiales, Aytoniaceae) and related genera (Long et al., 2000) and recent studies on *Plagiochila* (Jungermanniales, Plagiochilaceae) by Groth et al. (2002, 2003). These studies have proved very useful in inferring phylogenies at lower levels and, in the case of *Asterella*, demonstrating the paraphyly of a genus considered monophyletic on morphological criteria. Such studies are likely to increase in the future to shed light on some of the many problems of liverwort systematics.

The present study arose out of a taxonomic revision of the genus *Anastrophyllum* (Lophoziaceae) in the Sino-Himalayan region (Schill & Long, 2003). Using DNA-based inferences, it aims to investigate the phylogenetic relationships of selected species within the genus *Anastrophyllum*, to test its monophyly, and to look for relationships between *Anastrophyllum* and other genera of Lophoziaceae and other families of Jungermanniales sometimes regarded as related to Lophoziaceae, such as Jungermanniaceae, Gymnomitriaceae, and Scapaniaceae.

Materials and Methods

Taxon sampling

Material for DNA analyses (Table 1) was gathered by D. G. Long in Scotland, England, Nepal, and Bhutan and by D. G. Long and D. B. Schill in Norway. Specimens for DNA analysis were dried and stored in small polythene bags containing silica

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Table 1. Liverwort materials used for DNA analysis and GenBank accession numbers. Voucher specimens are deposited in E.

Taxon	Locality	Collection	GenBank accession no. *atp*B-*rbc*L	GenBank accession no. *trn*L
CALYPOGEIACEAE				
Calypogeia muelleriana (Schiffn.) Müll. Frib.	Scotland, Lanarkshire, Low Moss	*Long 29844*	AY453592	AY453769
DELAVAYELLACEAE				
Delavayella serrata Steph.	Nepal, Rasuwa District, between Trisuli Khola and Dimsa	*Long 30388*	AY453598	AY453775
GYMNOMITRIACEAE				
Marsupella emarginata (Ehrh.) Dumort.	Scotland, Aberdeenshire, Coire Kander	*Long 31293*	AY453593	AY453770
JUNGERMANNIACEAE				
Jungermannia exsertifolia Steph. subsp. *cordifolia* (Dumort.) Váňa	Scotland, West Sutherland, Gleann Dubh	*Long 29178*	AY453597	AY453774
Mylia taylorii (Hook.) Gray	Scotland, Peeblesshire, Talla Linns	*Long 31223*	AY453595	AY453772
Nardia compressa (Hook.) Gray	Norway, Sogn og Fjordane, Tornesstøl	*Long & Schill 31378*	AY453594	AY453771
LOPHOZIACEAE				
Anastrepta orcadensis (Hook.) Schiffn.	Scotland, Wester Ross, Inverpolly	*Long 29183*	AY453599	AY453776
Anastrophyllum saxicola (Schrad.) R. M. Schust.	Norway, Sogn og Fjordane, Mørkedalen	*Long & Schill 31371*	AY453610	AY453787
Anastrophyllum minutum (Schreb.) R. M. Schust.	Norway, Sogn og Fjordane, Branda-tjonna	*Long & Schill 31393*	AY453611	AY453788
Anastrophyllum hellerianum (Lindenb.) R. M. Schust.	Scotland, Perthshire, Finlarig Burn	*Long 29863*	AY453612	AY453789
Anastrophyllum donnianum (Hook.) Steph.	Scotland, Easterness, Glen Einich	*Long 29876*	AY453613	AY453790
Anastrophyllum joergensenii Schiffn.	Norway, Sogn og Fjordane, Endestadnipa	*Long & Schill 31400*	AY453614	AY453791
Barbilophozia floerkei (F. Weber & D. Mohr) Loeske	Scotland, Berwickshire, near Raecleugh	*Long 31215*	AY453607	AY453784
Lophozia ventricosa (Dicks.) Dumort.	Scotland, Berwickshire, Jordonlaw Moss	*Long 31226*	AY453600	AY453777
Jamesoniella nipponica S. Hatt.	Nepal, Rasuwa District, between Thulo Syabru and Brabal	*Long 30702*	AY453596	AY453773
Plicanthus hirtellus (F. Weber) R. M. Schust.	Bhutan, Bumthang District, Chume Chu	*Long 28602*	AY453608	AY453785
Tetralophozia setiformis (Ehrh.) Schljakov	Norway, Sogn og Fjordane, Mørkedalen	*Long & Schill 31365*	AY453609	AY453786
Tritomaria quinquedentata (Huds.) H. Buch	Scotland, Selkirkshire, Elibank Craig	*Long 29130*	AY453601	AY453778
SCAPANIACEAE				
Diplophyllum albicans (L.) Dumort.	England, Cornwall, Rusey Cliff	*Long 29024*	AY453602	AY453779
Douinia ovata (Dicks.) H. Buch	Scotland, Argyll, Allt Coire Gabhail	*Long 29808*	AY453603	AY453780
Scapania gracilis Lindb.	England, Cornwall, Rusey Cliff	*Long 29023*	AY453604	AY453781
Scapania ornithopodioides (With.) Waddell	Scotland, Argyll, Allt Coire Gabhail	*Long 29820*	AY453605	AY453782
Scapania undulata (L.) Dumort.	Scotland, Peeblesshire, Talla Linns	*Long 31219*	AY453606	AY453783

gel (Chase & Hillis, 1991). Voucher specimens are deposited in the herbarium at the Royal Botanic Garden Edinburgh (E). The study group (ingroup) included five species of *Anastrophyllum* (Spruce) Steph. and other available genera of Lophoziaceae (*Anastrepta* (Lindb.) Schiffn., *Barbilophozia* Loeske, *Jamesoniella* (Spruce) Carrington, *Lophozia* (Dumort.) Dumort., *Plicanthus* R. M. Schust., *Tetralophozia* (R. M. Schust.) Schljakov, *Tritomaria* Schiffn. ex Loeske) and Scapaniaceae (*Diplophyllum* (Dumort.) Dumort., *Douinia* (C. E. O. Jensen) Buch, and three species of *Scapania* (Dumort.) Dumort.).

Outgroup selection for molecular studies on the Jungermanniales is problematic, as no broad-based phylogeny including all major families of leafy liverworts has been published. However, the most up-to-date classification of leafy liverworts (Crandall-Stotler & Stotler, 2000) divides the order Jungermanniales into eight suborders, of which the Jungermanniaceae s.l. (including Lophoziaceae), Mesoptychiaceae, Gymnomitriaceae, and Scapaniaceae make up the suborder Jungermanniineae. Potential outgroups were selected from available genera of three families in the suborder Jungermanniineae: Jungermanniaceae s. str. (*Mylia* Gray, *Nardia* Gray, and *Jungermannia* L.), Gymnomitriaceae (*Marsupella* Dumort.), and Delavayellaceae (*Delavayella* Steph.). An additional potential outgroup was selected from outside this suborder, namely, *Calypogeia* Raddi, which belongs to the Calypogeiaceae in the suborder Lepidoziineae.

DNA EXTRACTION

Total genomic DNA was isolated from dried liverwort tissue (3–10 stems per individual) using a modified miniprep CTAB method of Doyle and Doyle (1990). Tissue samples were ground to a fine homogenate, using a glass pestle, and incubated at 65°C for 30 minutes in 2 X CTAB buffer, with the inclusion of 0.2% 2-mercaptoethanol and 0.1% polyvinylpolypyrrolidone. Two chloroform isoamyl alcohol extractions were performed. The DNA was precipitated using ice-cold isopropanol and the resultant DNA pellet was resuspended in 30–50 µl sterile, distilled water.

PCR AMPLIFICATION

The *trn*L intron was amplified using the primers of Frey et al. (1999) (*trn*L-c CGAAATTGGTAGACGCTGCG and *trn*L-d GGGGGTAGAGGGACTTGAAC), and the intergenic spacer between *atp*B and *rbc*L was amplified using the primers described by Chiang et al. (1998) (*atp*B-forward ACATCKARTACKGGACCAATAA and *rbc*L-reverse AACACCAGCTTTRAATCCAA). PCRs were performed in 25 µl reactions, including 1.25 mM $MgCl_2$, 400 nM of forward and reverse primer, 100 µM of each dNTP, 0.6 U *Taq* polymerase (Bioline, London, U.K.), and with approximately 10 ng DNA. The PCR cycling conditions used to amplify the *trn*L intron were: 94°C for 5 min. followed by 35 cycles of 94°C for 1 min., 50°C for 1 min., and 72°C for 1 min., with a final extension of 2 min. at 72°C. The *atp*B-*rbc*L spacer was amplified using the cycling conditions: 94°C for 2 min. followed by 30 cycles of 94°C for 45 sec., 57°C for 1 min., and 72°C for 3 min., with a final extension of 10 min. at 72°C. All cycling reactions were performed using a PTC-200 Peltier Thermal Cycler (M. J. Research, Waltham, Massachusetts, U.S.A.). PCR products were subsequently purified using a QIAquick Purification Kit (QIAGEN, Valencia, California, U.S.A.) according to the manufacturer's instructions.

Sequencing reactions were performed in a volume of 20 µl using a Thermo Sequenase Dye Terminator Sequencing Kit (Amersham, Piscataway, New Jersey, U.S.A.). Samples were run on an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, California, U.S.A.) and sequences were edited using Sequence Navigator (Applied Biosystems). Forward and reverse sequences were obtained for each cpDNA region to overcome editing ambiguities. GenBank accession numbers for each sequence are given in Table 1.

PHYLOGENETIC ANALYSIS

Matrix/sequence alignment

The alignment of the final matrix was adjusted manually by eye. The alignment for both the *trn*L intron and *atp*B-*rbc*L spacer was not unproblematic and ambiguous regions were present, particularly among the outgroup taxa, allowing different alignment interpretations. However, the ingroup taxa could be reliably aligned, except for *Jamesoniella.* Initial analyses were performed with exclusion of the ambiguous regions for both outgroup and ingroup taxa and with an exclusion of the ambiguous region only for the outgroups (off-set). (The sequence alignment matrices are available from the authors on request.) The tree topologies from both exclusion options were fully congruent, but the latter option was preferred as this allowed inclusion of informative sites in the offset region of the ingroup, resulting in higher resolved tree topologies. Separate analyses of the *trn*L intron and the *atp*B-

*rbc*L intergenic region were carried out as well as an analysis on the combined data.

Parsimony analysis

PAUP* version 4.0b10 (Swofford, 2002) was used for reconstruction of phylogenetic relationships based on maximum parsimony (Felsenstein, 1983). Both phylograms and strict consensus trees were obtained. Descriptive statistics were given by the consistency index (CI), homoplasy index (HI), retention index (RI), and rescaled consistency index (RC). All phylogenetic trees were created from unweighted, unordered characters. Gaps were treated as missing and multistate character states were interpreted as uncertain. For character optimization the option "accelerated transformation (ACCTRAN)" was used. Choice of one of the six potential outgroups for rooting the trees was determined by analyzing unrooted phylograms in PAUP* and the taxon with the longest branches was selected as root. Based on this unrooted tree, *Calypogeia muelleriana* (Calypogeiaceae) showed the longest branch length and was therefore chosen to root the trees.

All analyses were obtained by a heuristic search approach. Starting trees were obtained via stepwise addition. Heuristic searches were performed with 10,000 RANDOM addition sequence replicates using TBR with MulTrees on, STEEPEST DESCENT off and branches collapse if minimum branch length is zero. Bootstrap analyses were performed using PAUP* set to heuristic search, RANDOM addition sequence replicates, TBR on, and MulTrees and STEEPEST DESCENT off. Branch support was calculated as bootstrap values on the basis of 1000 replicates (Felsenstein, 1985) and decay indices (Bremer, 1988). Sequence characteristics (Table 2) were calculated using PAUP* or MacClade version 3.07 (Maddison & Maddison, 1992). The Partition Homogeneity Test was performed on 1000 replicates of RANDOM addition sequence using the same heuristic search settings as before.

Results

Sequence analyses

trn*L analysis*

Alignment of the 23 *trn*L sequences resulted in a matrix of 446 base pairs. Characteristic features of the matrix are given in Table 2. The alignment of all taxa required the insertion of several gaps due to indel events (insertions/deletions) and ambiguous parts were left out. Of the 360 unambiguously alignable sites included, 67% were constant. Of 33% variable characters, 16% were autapomorphies, unique to individual taxa and so parsimony uninformative, and 17% were informative. The transition to transversion ratio was 1.27 and is, hence, not biased. Sequence divergence in pairwise comparison was between 0.6% (*Anastrophyllum joergensenii* and *A. donnianum*) and 10.6% (*S. ornithopodioides* and *M. taylorii*) among all ingroup taxa; 0.6% (*A. joergensenii* and *A. donnianum*) and 5.9% (*L. ventricosa* and *Anastrophyllum hellerianum*) among Lophoziaceae, 2.1% (*S. undulata* and *S. gracilis*) and 6.3% (*Douinia ovata* and *Diplophyllum albicans*/*S. ornithopodioides*) among the Scapaniaceae, and 0.6% (*A. donnianum* and *A. joergensenii*) and 5.3% (*A. hellerianum* and *A. donnianum*/*A. joergensenii*) among *Anastrophyllum*. *Anastrophyllum joergensenii* differed from *A. donnianum* by only 2 base changes.

atp*B*-rbc*L analysis*

The alignment of the *atp*B-*rbc*L matrix showed a longer matrix length, with a total of 845 base pairs compared to the *trn*L matrix, and included a larger number of indels than *trn*L. Due to hypervariable regions within the spacer some data had to be excluded. The unambiguously aligned region included 619 characters of which 50% were constant. Of the 50% variable characters, 24% were parsimony uninformative. The transition/transversion ratio was 1.3, similar to the *trn*L data set. Sequence divergence in pairwise comparison was between 1.7% (*A. joergensenii* and *A. donnianum*) and 20.9% (*S. undulata* and *J. nipponica*) among ingroup taxa, 1.7% (*A. joergensenii* and *A. donnianum*) and 11.6% (*B. floerkii* and *Anastrepta orcadensis*) among Lophoziaceae, 3.4% (*S. ornithopodioides* and *S. gracilis*) and 10.6% (*S. undulata* and *D. ovata*) among Scapaniaceae, and between 1.7% (*A. donnianum* and *A. joergensenii*) and 6.3% (*A. saxicola* and *A. hellerianum*) for *Anastrophyllum*.

Phylogenetic analyses

trn*L intron sequence analysis*

The parsimony analysis resulted in 562 most parsimonious trees of 215 steps. The CI including uninformative characters was 0.55, RI 0.61, and, thus, the RC 0.41, which is low for a small matrix of 23 taxa and showed that many characters were homoplastic. Decay indices were up to 2 steps. The bootstrap values ranged from 56% to 98%. The strict consensus tree (not shown) showed only 5 internal branches within the study group. Monophyly of Scapaniaceae was supported by 69% bootstrap and a decay index of 1. Scapaniaceae and Lopho-

Table 2. Characteristics of *trn*L intron and *atp*B-*rbc*L intergenic region sequence matrices in offset,* based on alignment matrix excluding ambiguous regions.

Parameter	Program	*trn*L intron	*atp*B-*rbc*L	Combined
Number of taxa		23	23	23
Length range (total)(bp)	PAUP	305–372	562–626	906–97
Length mean (total)(bp)	PAUP	347.9	591.1	939
Length range (ingroup)(bp)	PAUP	305–372	562–611	906–960
Length mean (ingroup)(bp)	PAUP	348.4	586.4	934.8
Length range (outgroup)(bp)	PAUP	325–356	585–626	910–974
Length mean (outgroup)(bp)	PAUP	346	608	954
Aligned length (bp)	PAUP	446	845	1291
Aligned length (bp)—ambiguous	PAUP	360	619	978
G + C content, mean (%)	PAUP	22.9	32.8	26.6
Sequence divergence (Scapaniaceae)(bp)*	PAUP	7–21	18–56	29–71
Sequence divergence (Scapaniaceae)(%)*	PAUP	2.1–6.3	3.4–10.6	3.3–8.2
Sequence divergence (Lophoziaceae)(bp)*	PAUP	2–20	9–62	11–75
Sequence divergence (Lophoziaceae)(%)*	PAUP	0.6–5.9	1.7–11.6	1.3–8.6b
Sequence divergence (*Anastrophyllum*)(bp)*	PAUP	2–18	9–35	11–49
Sequence divergence (*Anastrophyllum*)(%)*	PAUP	0.6–5.3	1.7–6.3	1.3–5.5
Sequence divergence (ingroup)(bp)*	PAUP	2–30	9–87	11–114
Sequence divergence (ingroup)(%)*	PAUP	0.6–10.6	1.7–20.9	1.3–16.3
Sequence divergence (outgroup)(bp)*	PAUP	15–41	34–67	49–77
Sequence divergence (outgroup)(%)*	PAUP	5.2–14.7	7.9–15.6	6.8–10.8
Sequence divergence (in/outgroup)(bp)*	PAUP	16–44	71–93	98–137
Sequence divergence (in/outgroup)(%)*	PAUP	5.6–15.7	17.1–22.5	14.0–19.7
Number of variable sites (bp)*	PAUP	117	311	428
Number of variable sites (%)*	PAUP	33	50	44
Number of constant sites (bp)*	PAUP	241	307	548
Number of constant sites (%)*	PAUP	67	50	56
Number of informative sites (bp)*	PAUP	59	164	223
Number of informative sites (%)*	PAUP	17	27	23
Number of uninformative sites (bp)*	PAUP	58	147	205
Number of uninformative sites (%)*	PAUP	16	24	21
Transitions (minimum)*	MacCLADE	395	474	472
Transversions (minimum)*	MacCLADE	312	367	359
Transitions/Transversions*	MacCLADE	1.27	1.3	1.31
Length of most parsimonious tree(s), steps*	PAUP	215	585	811
Number of most parsimonious trees*	PAUP	562	1	1
Average number of steps per character*	PAUP	0.59	0.95	0.83
Consistency index (CI), including uninformative characters*	PAUP	0.55	0.6	0.57
Homoplasy index (HI), including uninformative characters*	PAUP	0.45	0.4	0.43
Retention index (RI)*	PAUP	0.61	0.72	0.68
Rescaled consistency index (RC)*	PAUP	0.41	0.51	0.47

ziaceae (subfamily Lophozioideae) monophyly was supported by 71% bootstrap. *Jamesoniella nipponica* and *Mylia taylorii* were sister to the Lophoziaceae/Scapaniaceae clade (bootstrap 80%, d = 2). *Delavayella serrata* was found outside the Lophoziaceae/Scapaniaceae clade. The five species of *Anastrophyllum* did not form a monophyletic clade. *Anastrophyllum joergensenii* and *A. donnianum* were the only species within the genus, which form a separate well-supported sister pair (bootstrap 98%, d = 4) and appeared to be sister to *Anastrepta orcadensis* but with low bootstrap support (bootstrap 56%, d = 1). This clade together with the Scapaniaceae, five other genera of Lophoziaceae (*Lophozia, Tritomaria, Barbilophozia, Plicanthus,* and *Tertralophozia*), and the remaining three species of *Anastrophyllum* fell in a polytomy.

atp*B*-rbc*L spacer sequence analysis*

The parsimony analysis resulted in one single most parsimonious tree of 585 steps length (figure

not shown). CI including uninformative characters was 0.60, RI 0.72, and RC 0.51. The decay indices for each clade were between 1 and 12 steps and the bootstrap values ranged from 52% to 100%. The single most parsimonious tree was fully resolved. Although there was more resolution than in the *trn*L topology, the tree was congruent with the *trn*L topology (see below) but had higher branch support (bootstrap and decay index). Lophoziaceae subfamily Lophozioideae and Scapaniaceae were monophyletic (bootstrap 100%, d = 9). The Scapaniaceae appeared as a monophyletic group (bootstrap 100%, d = 9). The Lophoziaceae were paraphyletic with its subfamily Lophozioideae split into two major clades: Lophozioideae I (bootstrap 85%, d = 4) and Lophozioideae II (bootstrap 93%, d = 7). *Delavayella serrata* was found to be outside the Scapaniaceae. Paraphyly of *Anastrophyllum* was supported by a high bootstrap value (92%) and decay index (d = 6). *Anastrophyllum joergensenii* and *A. donnianum* (bootstrap 98%, d = 7) and *A. saxicola* and *A. minutum* (bootstrap 99%, d = 8), each forming sister pairs, appeared as very closely related taxa, which share a common ancestor.

Combined trn*L intron and* atp*B*-rbc*L sequence analysis*

The Partition Homogeneity Test showed no significant incongruence between the two data sets (0.089), so the two data sets could be combined. The combined phylogenetic analysis resulted in a single most parsimonious tree with a similar topology to that of the *atp*B-*rbc*L tree (Fig. 1). The tree length was 811 steps. The CI including uninformative characters had a value of 0.57, RI was 0.68, and RC 0.47. Decay indices were between 1 and 16 steps. The bootstrap values ranged from 50% to 100%. It showed an even higher decay index for the paraphyly of Lophoziaceae (d = 16) and the monophyly of Scapaniaceae (d = 16). The Scapaniaceae were more closely related to Lophozioideae I (*Tritomaria/Anastrepta/Lophozia* clade) than to the Lophozioideae II (*Plicanthus/Barbilophozia/Tetralophozia/Anastrophyllum* clade). *Barbilophozia floerkii* and *Lophozia ventricosa* were found in separate clades within the subfamily. *Jamesoniella nipponica* (Lophoziaceae subfamily Jamesonielloideae) was sister to the rest of the Lophoziaceae/Scapaniaceae clade (bootstrap 89%, d = 5). Differences from the *atp*B-*rbc*L analysis are that *A. hellerianum* and *Plicanthus hirtellus* swap positions, *Diplophyllum albicans* and *Douinia ovata* now form a sister pair (bootstrap 68%, d = 2), and the five *Anastrophyllum* species appear to be monophyletic, albeit with low branch support (< 50%, d = 1).

Discussion

Suitability of *trn*L intron and *atp*B-*rbc*L intergenic region

In the present study both chloroplast regions show a high rate of evolutionary change and hence are evolving very fast, as shown by the large number of gaps. As a result, outgroup taxa in particular were particularly difficult to align, whereas most ingroup taxa and particularly sequences of *Anastrophyllum* were rather well conserved showing similarities over wide regions. Though the *atp*B-*rbc*L spacer was found to be very suitable for the study of relationships between closely related taxa, the unambiguous alignment of outgroup and some ingroup members was rather difficult. However, the ambiguous parts of the sequence have been excluded, and therefore possible ambiguous information could not influence the analysis. But despite this exclusion of potentially informative data, the analysis, which resulted in one fully resolved most parsimonious tree, proved that the spacer contained enough information to disentangle relationships supporting a single tree topology with significant branch support (> 80%).

The suitability of the *trn*L intron for alignment and phylogeny of liverworts has already been successfully tested in other studies (Long et al., 2000; Wheeler, 2000). However, both noted that ambiguous regions were present in their *trn*L matrix, which also had to be excluded before analysis. The intron analysis of the present study resulted, after exclusion, in several hypotheses of possible tree topologies, which did not contribute to elucidating phylogenetic relationships. The *atp*B-*rbc*L matrix showed, with 27%, a higher number of informative characters compared to the *trn*L matrix, indicating a higher rate of evolution than the former. The sequence difference for the *atp*B-*rbc*L spacer was in general higher than for the *trn*L intron. Indices for descriptive tree statistics, reflecting the amount of phylogenetic signal in the analysis, were in the *atp*B-*rbc*L analysis higher than in the *trn*L analysis indicating more homoplasy in the latter.

Phylogenetic and taxonomic implications

Because the study was focused around the relationships of *Anastrophyllum*, Lophoziaceae, and Scapaniaceae, the combined analysis (Fig. 1) provides valuable insight into the relationships of those taxa, but the limited sampling restricts other

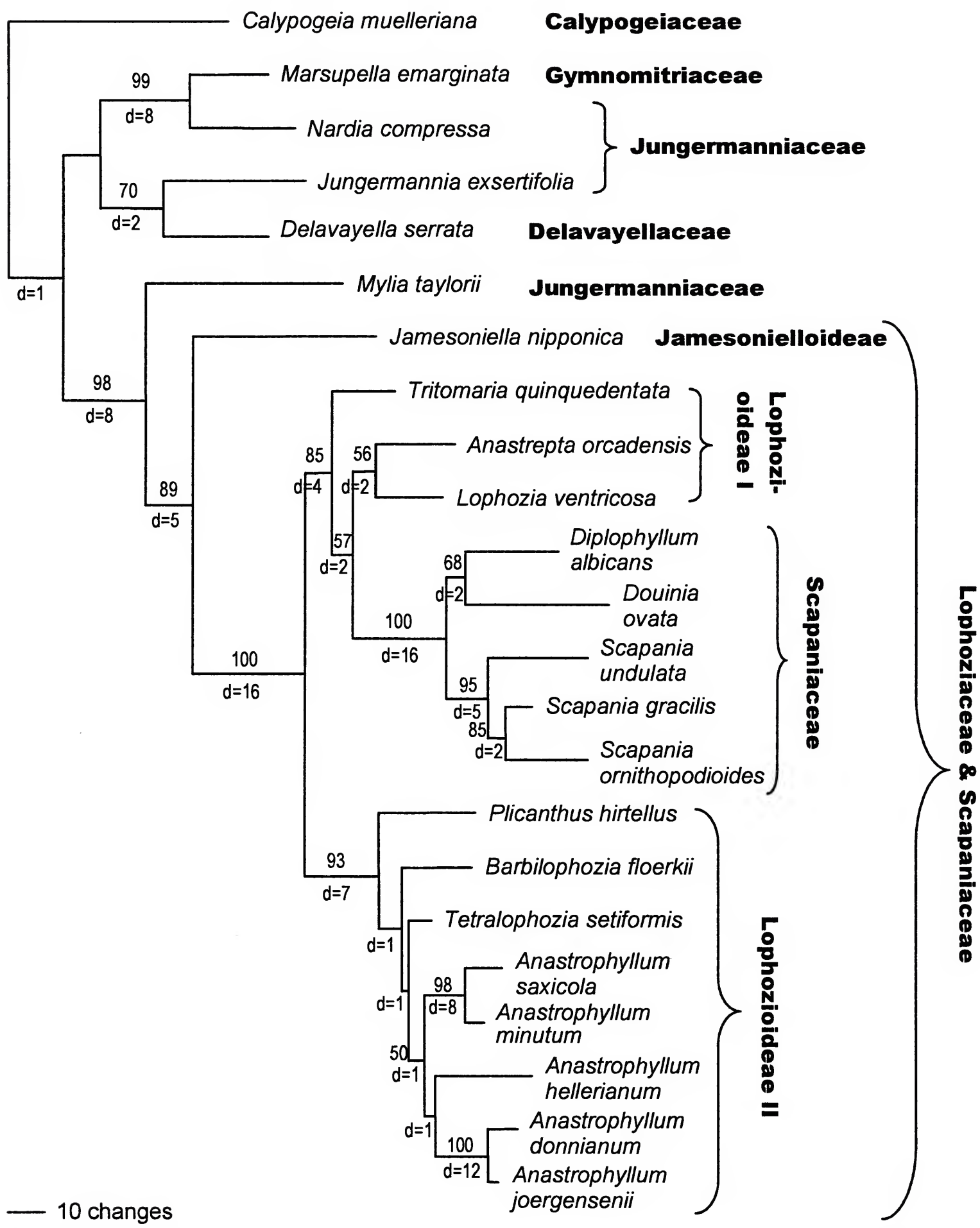

Figure 1. Single most parsimonious tree (length = 811 steps; CI = 0.57; RI = 0.68; RC = 0.47) based on combined *trn*L and *atp*B-*rbc*L sequences from 23 taxa with a heuristic search using unweighted parsimony. Numbers above branches indicate bootstrap support; numbers below branches indicate decay indices.

meaningful conclusions. Nevertheless, the exclusion of *Delavayella* from Scapaniaceae, as advocated by Schuster (1999) and Potemkin (1999), is supported, though no clues as to its closest relatives are given. Likewise, the relationships of the families Jungermanniaceae, Lophoziaceae, and Gymnomitriaceae, often placed together in suborder Jungermanniineae, as by Crandall-Stotler and Stotler (2000), are not clarified due to sampling limitations. Within the Lophoziaceae, the established separation of subfamilies Jamesonielloideae and Lophozioideae (Váňa, 1996; Grolle & Long, 2000) is well supported (85%, d = 4) with these appearing as sister groups. However, the most striking re-

sult of the study is the nesting of the Scapaniaceae within the subfamily Lophozioideae, contrary to all recent classifications, though they have sometimes been placed in the same suborder Jungermannineae (Crandall-Stotler & Stotler, 2000).

Division of Lophozioideae into two separate clades Lophozioideae I and Lophozioideae II has not been predicted by any published morphological study and, indeed, separates the two genera *Lophozia* and *Barbilophozia*, often treated as a single broad genus *Lophozia* (e.g., Schuster, 1969). *Lophozia* would appear to be closer to *Anastrepta*, and *Barbilophozia* to *Tetralophozia* and *Anastrophyllum*. The Scapaniaceae, nevertheless, remains monophyletic, but if recognized as a family distinct from Lophoziaceae, the latter family becomes paraphyletic. Nomenclaturally, the earlier family name would be Scapaniaceae. It is considered premature to subsume Lophoziaceae into that family without further research. Equally, the split of Scapaniaceae by Potemkin (1999) into Diplophyllaceae (including *Diplophyllum* and *Douinia*) and a monogeneric Scapaniaceae cannot be justified without sampling a wider range of *Scapania* species to include some that may be closely allied to *Diplophyllum*. In a concurrent study by Yatsentyuk et al. (2004 this issue), using only *trn*L-*trn*F sequences of a similar but broader range of samples, the same nesting of Scapaniaceae in Lophoziaceae, and a similar segregation of the genera of Lophoziaceae, was obtained.

RELATIONSHIPS BETWEEN THE LOPHOZIACEAE AND SCAPANIACEAE

As pointed out by Potemkin (1999), close links between the Lophoziaceae and Scapaniaceae have been suggested several times in the past, perhaps first by Spruce (1885–1886: 418) in comparing *Sphenolobus* (Lophoziaceae) and *Diplophyllum* (Scapaniaceae): "it is a merely artificial line which we are obliged to draw between two adjacent groups." This was developed by Buch (1928), who postulated that *Scapania* species had derived from *Lophozia*-like or *Sphenolobus*-like ancestors. Schuster (1951: 10) concurred with this view: "The Scapaniaceae appear to have been directly derived from *Anastrophyllum*- or *Tritomaria*-like ancestral forms in the Lophoziaceae." This statement is largely supported by the present study. Schuster (1969, 1974) reiterated these ideas; yet, the recent debate has been dominated by the suggested links between Lophoziaceae and Jungermanniaceae, which have often been united as a single broad Jungermanniaceae (e.g., Schuster, 2002). On the other hand, the close links suggested between Lophoziaceae and Scapaniaceae have not been directly reflected in published classifications and Scapaniaceae has been consistently kept apart from these two, sometimes with the Gymnomitriaceae and other families placed in between (e.g., Schuster 1969, 1974; Long & Grolle, 2000). Further molecular evidence is needed to evaluate the relationships among Lophoziaceae, Jungermanniaceae, and Gymnomitriaceae, but the present study suggests that if any one family is to be considered for union with Lophoziaceae, Scapaniaceae is that family.

MONOPHYLY OF *ANASTROPHYLLUM*

Monophyly of *Anastrophyllum* is suggested by this study but is not well supported (< 50%) and hence should be considered doubtful. The concurrent study by Yatsentyuk et al. (2004) places *Barbilophozia kunzeana* within the *Anastrophyllum* clade, suggesting that broader sampling may undermine this apparent monophyly of *Anastrophyllum*. However, the species *A. saxicola* and *A. minutum* are closely related to each other, as are *A. donnianum* and *A. joergensenii*, and the latter relationship is well supported morphologically (Schill & Long, 2003).

CONCLUSIONS

The two separate analyses as well as the combined analysis all suggest that the Lophoziaceae is a paraphyletic family with Scapaniaceae nested within it and having evolved from the subfamily Lophozioideae in a single evolutionary event. However, it is considered premature to merge the two families into a single enlarged Scapaniaceae. The relationships of Jungermanniaceae and Gymnomitriaceae to Lophoziaceae are not resolved due to sampling limitations, but are clearly not as close as that of Scapaniaceae. The status of *Anastrophyllum* as a monophyletic genus has very low branch support and is in conflict with the results of both the *trn*L and *atp*B-*rbc*L analyses and must therefore be considered doubtful. *Delavayella* should be kept out of the Scapaniaceae and retained in its own family Delavayellaceae.

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ON PHYLOGENY OF LOPHOZIACEAE AND RELATED FAMILIES (HEPATICAE, JUNGERMANNIALES) BASED ON *TRN*L-*TRN*F INTRON-SPACER SEQUENCES OF CHLOROPLAST DNA[1]

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Abstract

The sequences of the *trn*L intron and 3′ exon and *trn*L-*trn*F intron and spacer of the chloroplast DNA were studied in 47 hepatic species from Lophoziaceae (24 species) and fewer representatives of Scapaniaceae (7 spp.), Diplophyllaceae (3 spp.), Jungermanniaceae (7 spp.), Gymnomitriaceae (4 spp.), Mesoptychiaceae (1 sp.), and Cephaloziaceae (1 sp.). Analyses by parsimony, neighbor-joining, Bayesian, and metapopulation genetic algorithm gave similar results. Scapaniaceae are monophyletic and form a terminal clade within Lophoziaceae, whereas the latter (excluding *Leiocolea* and *Hattoriella*) form a paraphyletic grade. *Leiocolea rutheana* is found closely related to *Mesoptychia,* and species of *Leiocolea* and *Mesoptychia* form a well-supported clade nested within the Jungermanniaceae in parsimony and neighbor-joining analyses. Present results support the usage of a narrow generic concept in Lophoziaceae and Jungermanniaceae.

Key words: chloroplast DNA, Gymnomitriaceae, Hepaticae, Jungermanniaceae, *Leiocolea,* Lophoziaceae, *Mesoptychia,* phylogeny, Scapaniaceae, *trn*L-*trn*F.

Historic classification of the order Jungermanniales has been complicated and remains controversial (Table 1). The limits of families and genera accepted by different authors has been inconsistent. For example, the genus *Lophozia* sensu Schuster (1969, 2002) includes eight genera according to the concept of Schljakov (1980): *Lophozia* (Dumort.) Dumort. s. str., *Barbilophozia* Loeske, *Orthocaulis* H. Buch, *Protolophozia* (R. M. Schust.) Schljakov, *Schistochilopsis* (Kitag.) Konstantinova (= *Massula* Schljakov, *Massularia* Schljakov), *Obtusifolium* S. W. Arnell, *Isopaches* H. Buch, and *Leiocolea* (Müll. Frib.) H. Buch. Other recent European authors adopted intermediate concepts, recognizing *Lophozia*, *Barbilophozia*, and *Leiocolea*, but not the remaining genera (Paton, 1999; Grolle & Long, 2000).

In recent years DNA sequence data have significantly contributed to bryophyte systematics and phylogenetics. After an initial period resolving the relationships among major lineages of bryophytes at suprafamilial level (cf. Kenrick & Crane, 1997; Lewis et al., 1997; Hedderson et al., 1998; Samigullin et al., 1998, 2002; Wheeler, 2000; and references cited therein), the systematics of particular families is becoming a main focus for studies. This had led to more natural classifications at the family and genus levels both in mosses (Goffinet & Vitt, 1998; Hyvönen et al., 1998, 2004; Shaw, 2000; Goffinet et al., 2001; Ignatov & Huttunen, 2002; Pedersen & Hedenäs, 2002; Vanderpoorten et al., 2002; Cox & Hedderson, 2003) and to a lesser extent in hepatics (He-Nygrén & Piippo, 2003; Schill et al., 2004). Several of these aforementioned stud-

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Table 1. Taxonomic treatments of genera of Jungermanniales.

Schuster (1969)	Schuster (2002)	Grolle & Long (2000)	Paton (1999)	Schljakov (1980, 1981), Konstantinova et al. (1992)
Chandonanthus subgen. *Tetralophozia*	*Tetralophozia*	*Tetralophozia*	*Tetralophozia*	*Tetralophozia*
Lophozia subgen. *Barbilophozia*	*Lophozia* subgen. *Barbilophozia*	*Barbilophozia* subgen. *Barbilophozia*	*Barbilophozia*	*Barbilophozia*
Lophozia subgen. *Orthocaulis*	*Lophozia* subgen. *Orthocaulis*	*Barbilophozia* subgen. *Orthocaulis*	*Barbilophozia*	*Orthocaulis*
Lophozia subgen. *Lophozia*	*Lophozia* subgen. *Lophozia*	*Lophozia* subgen. *Lophozia*	*Lophozia*	*Lophozia*
Lophozia subgen. *Protolophozia*	*Lophozia* subgen. *Protolophozia*	*Lophozia* subgen. *Protolophozia*	*Lophozia*	*Protolophozia*
Lophozia subgen. *Massula*	*Lophozia* subgen. *Schistochilopsis*	*Lophozia* subgen. *Schistochilopsis*	*Lophozia*	*Massula (Massularia)* = *Schistochilopsis*
Lophozia subgen. *Massula* sect. *Obtusae*		*Lophozia* subgen. *Schistochilopsis* sect. *Obtusae*	*Lophozia*	*Obtusifolium*
Lophozia subgen. *Isopaches*	*Lophozia* subgen. *Isopaches*	*Lophozia* subgen. *Isopaches*	*Lophozia*	*Isopaches*
Lophozia subgen. *Leiocolea*		*Leiocolea*	*Leiocolea*	*Leiocolea*
Anastrophyllum subgen. *Sphenolobus*	*Anastrophyllum* subgen. *Sphenolobus*	*Anastrophyllum* subgen. *Sphenolobus*	*Anastrophyllum*	*Sphenolobus* subgen. *Sphenolobus*
Anastrophyllum subgen. *Crossocalyx*	*Anastrophyllum* subgen. *Crossocalyx*	*Anastrophyllum* subgen. *Crossocalyx*	*Anastrophyllum*	*Crossocalyx*
Anastrophyllum subgen. *Eurylobus*		*Anastrophyllum* subgen. *Eurylobus*	*Anastrophyllum*	*Sphenolobus* subgen. *Eurylobus*
Anastrophyllum subgen. *Anastrophyllum*	*Anastrophyllum* subgen. *Anastrophyllum*	*Anastrophyllum* subgen. *Anastrophyllum*	*Anastrophyllum*	*Anastrophyllum* subgen. *Anastrophyllum*
Anastrophyllum subgen. *Schizophyllum*	*Anastrophyllum* subgen. *Schizophyllum*	*Anastrophyllum* subgen. *Schizophyllum*	*Anastrophyllum*	*Anastrophyllum* subgen. *Schizophyllum*
Jungermannia		*Jungermannia* subgen. *Liochlaena*	*Jungermannia*	*Liochlaena*
Tritomaria subgen. *Tritomaria*	*Tritomaria* subgen. *Tritomaria*	*Tritomaria* subgen. *Tritomaria*	*Tritomaria*	*Tritomaria* subgen. *Tritomaria*
Tritomaria subgen. *Tritomaria* sect. *Trilophozia*	*Tritomaria* subgen. *Trilophozia*	*Tritomaria* subgen. *Tritomaria* sect. *Trilophozia*	*Tritomaria*	*Tritomaria* subgen. *Tritomaria* sect. *Trilophozia*
Tritomaria subgen. *Saccobasis*		*Tritomaria* subgen. *Saccobasis*	*Tritomaria*	*Saccobasis*
Solenostoma subgen. *Solenostoma*	*Solenostoma* subgen. *Solenostoma*	*Jungermannia* subgen. *solenostoma*	*Jungermannia*	*Solenostoma*
Solenostoma subgen. *Luridae*	*Jungermannia*	*Jungermannia* subgen. *Jungermannia*	*Jungermannia*	*Jungermannia*
Solenostoma subgen. *Plectocolea*	*Jungermannia* subgen. *Plectocolea*	*Jungermannia* subgen. *Plectocolea*	*Jungermannia*	*Plectocolea*
Diplophyllum subgen. *Macrodiplophyllum*				*Macrodiplophyllum*

ies have proved the usefulness of the *trn*L-*trn*F region sequences to bryophyte systematics, and in this paper we investigate if sequence information of this region can help to resolve problems within the classification of Lophoziaceae.

Methods

Selection of taxa

The main focus of this study was the family Lophoziaceae, represented in our analysis by 24 species. We tried to include most genera (and from larger genera also distinct infrageneric taxa) from the boreal zone of the Northern Hemisphere for which we had recently collected material available. Two species were taken for each genus when possible. One species, *Orthocaulis kunzeanus*, was sampled by two specimens because preliminary analysis revealed that it is distinct from the other species of this genus (however, the second specimen appeared identical to the first one in its *trn*L-*trn*F sequence). Taxa from putatively related families from Jungermaniales suborder Jungermanniinae were also included: Scapaniaceae (7 spp.), Diplophyllaceae (3 spp.), Jungermanniaceae (7 spp.), Gymnomitriaceae (4 spp.), and Mesoptychiaceae (1 sp.). *Hygrobiella laxifolia* was used as the outgroup for all analyses, as this species belongs to a different suborder, Jungermanniales suborder Cephaloziineae Schljakov (cf. Konstantinova et al., 1992). Species used in analyses are listed in Appendix 1.

DNA isolation, PCR amplification, and DNA sequencing

Total genomic DNA was extracted from herbarium specimens by the CTAB method (Doyle & Doyle, 1987) or by using NucleoSpin Plant Kit (MACHEREY-NAGEL, Düren, Germany). The cpDNA *trn*L_{UAA}-*trn*F_{GAA} region that includes the *trn*L intron and the *trn*L-*trn*F intergenic spacer (IGS) was amplified and sequenced using primers C and F that correspond to conserved sequences near the 5′ and 3′ ends of tRNA genes (Taberlet et al., 1991). Purified DNA samples were subjected to automatic or manual sequencing.

For automatic procedure PCR amplifications were performed on PTC-100 thermal cycler (M. J. Research, Waltham, Massachusetts, U.S.A.) with the following protocol: 10 min. at 95°C, 35 cycles (1 min. at 95°C, 1 min. at 49°C, 1 min. at 72°C), and a 7 min. at 72°C extension time after completion of the 35 thermal cycles. The PCR products were visualized on 1% agarose Q-BIO Gene TBE gels and purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, New Jersey, U.S.A.) and then used as templates in sequencing reactions with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, U.S.A.). PCR products were analyzed on an automated ABI PRISM 377 DNA Sequencer.

For manual sequencing the PCR protocol was performed on Tercik thermocycler (DNA Technology, Russia) with the following protocol: 3 min. at 95°C, 30 cycles (50 sec. At 94°C, 40 sec. at 50°C, 60 sec. at 72°C) and a 7 min. at 72°C extension time. PCR products were purified using purification columns (QIAGEN, Valencia, California, U.S.A.) and sequenced employing Cycle Reader DNA Sequencing kit (Fermentas, Lithuania) with α-^{33}P-dATP. Nucleic sequences were resolved on 6% polyacrylamide gels. All PCR products were sequenced using two primers.

The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers indicated in Appendix 1.

Phylogenetic analyses

Sequences were aligned manually using the SED editor of the VOSTORG package (Zharkikh et al., 1990).

For the phylogenetic analyses five analytical procedures were implemented: (1) neighbor-joining method with the program TREECON; (2) parsimony analyses with the program PAUP*; (3) parsimony analyses with the program Nona; (4) Bayesian analysis with the program MrBayes; and (5) metapopulation genetic algorithm with the program MetaPIGA.

A neighbor-joining (NJ) method was employed (Saitou & Nei, 1987) using the TREECON 1.3b package (Van de Peer & De Wachter, 1997). Construction of distance matrices was done using uncorrected pairwise sequence divergence, *p*-distances (Van de Peer & De Wachter, 1997), Kimura's two-parameter model distances (Kimura, 1980) and Galtier and Gouy (1995) distances. Gaps were taken into account using an option treating contiguous gaps as one gap. Support for individual nodes was assessed using a bootstrap resampling procedure with 1000 replicates (Felsenstein, 1985).

Parsimony (P) analyses were performed with the programs PAUP* 4.0b10 (Swofford, 1998) and Nona (Goloboff, 1993), the latter in conjunction with Winclada shell (Nixon, 2002). Analyses with PAUP* involved a heuristic search with TBR (tree-bisection-reconnection) branch swapping with MulTrees, and Collapse options in effect. Charac-

ters were unordered, no weighting scheme was employed, and random sequence addition was used. Gaps were treated as an additional character state (fifth base) and bases coded as totally ambiguous (N) were treated in both analyses as X (any nucleotide or a gap). One hundred jackknife resamplings (Penny & Hendy, 1986) were performed with 10% of the data to be deleted for each replicate, and a majority rule consensus tree was constructed. Nona analyses were performed with the following settings: hold * (holding all trees that memory allows; in current settings with WinClada this is 10,000); mult*1000 (search replicated 1000 times); hold/10 (keeping 10 starting trees for each replication) and using the multiple TBR algorithm (mult*max).

The best method for including alignment gaps in phylogenetic analysis is a matter of dispute. While we acknowledge that length variable regions do contain valuable information, it is not clear whether treating each individual gap as a character (fifth base) is the best approach. One can also argue for treating contiguous gaps as a single character. In order to downweigh the effect of alignment gaps, we also performed what can be called a conservative or cautious analysis treating gaps as missing data. This conservative analysis was also performed with Nona with the settings described above.

Bayesian analysis was performed using MrBayes 2.01 computer program (MrB) (Huelsenbeck & Ronquist, 2001). GTR and HKY85 models of nucleotide substitution and invgamma model for among-site rate variation were used. Four Metropolis-coupled MCMC chains were run from a randomly chosen starting trees for 1,000,000 generations, trees were saved once every 10 generations, the first 9000 trees were ignored, and the 50% majority-rule consensus tree and Bayesian posterior probabilities as branch support values were calculated.

The metapopulation genetic algorithm (metaGA) for inferring phylogeny as implemented in the MetaPIGA 1.0.2b (MPG) computer program (Lemmon & Milinkovictch, 2002) was used with the following options: HKY85 model of evolution with invariable sites, two substitution types estimated from the data, three rate categories, five populations, 50 replications, the consensus pruning enabled, group consensus = strict; while other options were maintained as default values. As a result 250 best trees were saved and the majority rule consensus tree and metaGA posterior branch support values (that are close to MrBayes posterior probabilities) were calculated using PAUP* 4.0b10.

The negative log likelihood scores (−lnL) for phylogenetic trees constructed by MPG and MrB methods were calculated using PAUP* 4.0b10 software.

To test the homogeneity of substitution patterns between sequences disparity indices, I_D, (Kumar & Gadakhar, 2001) were calculated using MEGA version 2.1 computer software (Nei & Kumar, 2000).

Results

The entire alignment of 48 sequences consisted of 526 sites; the length of sequences varied from 373 to 442 nucleotides. Positions 303–325, 335–358, and 365–375 were excluded from the analysis because of their ambiguous alignments. The final analyzed alignment of 362–419 nucleotide-long sequences consisted of 468 sites (submitted to TreeBase, ⟨http://www.treebase.org⟩). Among these, 181 characters were constant, 81 variable characters were parsimony-uninformative, and 206 characters were parsimony-informative. Four parts of the alignment rich in gaps are presented in Table 2. The average nucleotide composition of a studied region (coding DNA chain) was 32.7% (A), 34.9% (T), 17.4% (C), and 15.0% (G).

The pairwise disparity index test revealed 89 pairs of sequences for which at 5% confidence level the hypothesis of homogeneity of substitution patterns has to be rejected. For particular sequences the number of paired sequences with higher confidence levels varied from 1 to 21. The most deviant sequences were found in *Hattoriella diversiloba* (21), *Obtusifolium obtusum* (16), *Leiocolea heterocolpos* (13), *Tritomaria exsecta* (10), and *Sphenolobus minutus* (7).

MPG trees were constructed first for the entire set of 48 sequences and then for subsets that excluded one to four sequences that demonstrated the greatest deviation from homogeneity of the evolution process and/or having the lowest branch support values. All trees with excluded sequences had identical or similar topologies to those including all exemplars, however, with differences in support values. Finally, only one species was pruned in a final MPG tree reconstruction presented in Figure 1. That is *Obtusifolium obtusum*, which had the second largest value of deviation from homogeneity of substitution patterns after *Hattoriella diversiloba*. This MPG tree with a log likelihood score -lnL = 3811.81 had the highest mean support value (90%) among other MPG trees with other variants of species pruning. Six major groups of species were revealed on the tree and marked as groups A–F. The pruning of the *Hattoriella diversiloba* showed no effect on the tree topology but resulted in 15%–20%

Table 2. Four parts of 526 bp alignment rich in indels. The characters are from coding DNA chain in the following order: 5′—*trn*F—intergenic spacer—*trn*L 3′ exon—*trn*L intron—3′. Numeration starts from 20th position from 3′ end of *trn*F. N—totally ambiguous character.

	188 ↓ 234 ↓ 361 ↓ 407 ↓
Scapania subalpina	*AA-----AT**ATGTCATTTATATAT--TTA-TATGTA-TATAT------AAAATT-CATATTAGAACGAACTAATAATAAA----TT-TA---TTTTT**TA-------AAACAAA**TTATTATC-CTCA*
Scapania undulata	*AA-----AT**ATATCATATATATAT--TTA-AATATA-TATT-------AAAATT-CATCGTAGAACGAACTAATAATCA-----TT-TA---TTTTT**TA-------AAAGAAA**TTATTATC-CTCA*
Scapania crassiretis	*AA-----AT**ATATCATATATATAT--TTA-AATATA-TAT--------AAAATT-CATCGTAGAACGAATTAATAATAAA-TGATT-TA---TTTTT**TA-------AAGGAAA**TTATTCTC-CTCA*
Scapania paludicola	*AA-----AT**ATATCATATGTATATATTTT-AATATA-TATT-AATATAAAAATT-CATCGTAGAACGAACTAATAATAAA-TAATT-TA---TTTTT**TA-------AAGGAAA**TTATTTTC-CTCA*
Scapania ferruginea	*AA-----AT**ATATAATATATATAT--TTA-AATATA-TT---------AAAATT-CATCGTAAAACGAACTAATAATAAA-TTATT-TA---TTTTT**TA-------AAGGAAA**TTATTCTC-CTCA*
Macrodiplophyllum microdontum	*AA-----AT**ATATCATAT--AT----TTA-AATATA-TA---------AAAATT-CATCGTAGAACGAATTAATAATTAA-TAATT-AA---TTTTT**TA-------AAGGAAA**TTATTCTC-CTCA*
Douinia ovata	*AA-----AT**ATATCATATATAT----TTA-AATATG-TA---------NAAATT-CATTGTAGAACGAACTAATAATAAA-TAA-T-NT---TTTNT**TA-------AGGGAAA**TTATCTTC-CGCA*
Macrodiplophyllum plicatum	*AA-----AT**ATATCATATATAT----TTA-AATATA-TA---------AAAATT-CATTGTAAAACGAACTAATAATAAA-TAATT-T----TTTTT**TA-------AGGGAAA**TTATTTTC-TGCA*
Diplophyllum obtusifolium	*AA-----AT**ATACTGT-TCTAT----TTA-AATATA-GA---------AAAATTTCATTGTAAAACGAACTAATAAT-----AATT-TA---TTTTT**TG-------AGGGAAA**TTATTCCT-CTCA*
Diplophyllum taxifolium	*AA-----AT**ATACTGT-TATATT---TAA-TATATA-TA---------TAAATT-CATTGTAAAACGAACTAATAATAAA-TAATT-CA---TTTTT**TG-------AAGGAAA**TTATTCCT-CTCA*
Schistochilopsis incisa	*AA------T**AT----------TT-CATTACAAAACGAACTAATAATAAA-TAATT-CA----TTTT**TA-------AGGGGAA**TTATTCTT-CTCA*
Schistochilopsis opacifolia	*AA------T**AT----------NT-CATTACAAAACGAACTAATAATAAA-TAATT-CA----TTTT**TA-------AGGGGAA**TTATTCTT-CTCA*
Lophozia longidens	*AA-----AT**ATCTTAT-TATATA---TAA-AATATA-TA---------AAAATT-CATTATAAAACGAATTAATATTAAA-TAATT-CA---TTTTT**TA-------AAGGAAA**TTATTTTT-CTCA*
Lophozia silvicola	*AA-----AT**ATTT----TATA-----TAA-AATATA-AT---------AAAATT-CATTATAAAACGAAGTAATTAT--------T-CA---TTTTT**TA-------AAGGAAA**TTATTCTT-CTCA*
Tritomaria quinquedentata	*AA-----AT**ATCCCAT-TATAT----TTA-AATATA-AT---------NAAATT-CATTATAAAACAAATTAATAATAAA-TAATT-CA---TTTTT**TA-------AAGTAAA**TTATTCTT-CTCA*
Tritomaria exsecta	*AA-----AT**ATCCCAT-TATATN---TTA-TATATA-AT---------NAATNT-CATTGTAAAACAAANTAATAATAAA-TAATT-CA---TTTTT**TA-------AAGTAAA**TTATTCTC-CTCA*
Saccobasis polita	*AA-----AT**AT---------------TTA-AGTAGA-A---------AAAATT-TATAATAAAACGAAATAATAATAAA-TAATA-TA---TTTTT**CA-------AAATAAG**TTATCCTT-CTCA*
Barbilophozia hatcheri	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTGTAGAACGAACTAATAATAAA-TAAAA-AA---TTTTT**TA-------AAGTAAA**TCATTCTT-CTCA*
Barbilophozia barbata	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTGTAAAGTGAACTAGTCACAAA-TAAAA-AA---TTTTT**TA-------AAGTAAA**TCATTCTT-CTCA*
Tetralophozia setiformis	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-NATTGTAAAACGAACTAATAATAAA-TAAAA-AA---TTTTT**TA-------AAGTAAA**TCATTCTT-CTCA*
Lophozia sudetica	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AGAATT-CATTATAAAACGAACTAATAATAAA----AA-AA----TTTT**TA-------AAGTAAA**TCATTCTT-CTCA*
Sphenolobus minutus	*AA-----AT**ATCTAATAT-TAT----TTA-AATATAAT---------AAAATT-CATTGTAAAACGAACTAATAATAAA-TAAAA-AA---TTTTT**TA-------AAGTAAA**TTATTCTT-CTCA*
Sphenolobus saxicola	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTGTAAAACGAAGTAATAATAAA-TAAAA-AA---TTTTT**TA-------AAGTAAA**TTATTCTT-CTCA*
Orthocaulis kunzeanus 1	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTTTTAAACGAACTAATAATAAATTAAAA-AA---TTTTT**TA-------AAGTAAA**TTATTCTT-CTCA*
Orthocaulis kunzeanus 2	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTTTTAAACGAACTAATAATAAATTAAAA-AA---TTTTT**TA-------AAGTAAA**TTATTCTT-CTCA*
Crossocalyx hellerianus	*AA-----AT**ATTTCATAT-TAT----TTA-AATATAAT---------GAAATCTCATTAGAAAACGAACTCATAATAAA-TAAAA-AA----TTTT**CA-------AAGTAAA**TTATTCTT-CTCA*
Anastrophyllum michauxii	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------GAAATT-CATTGTGAAACGAACTAATAATAAA-TAAAA-AA---TTTTT**TG-------AAGTAAA**TTATTCTT-CTCA*
Orthocaulis attenuatus	*AA-----AT**ATCTCATAT-TAT----TTA-AATCTAAT---------AAAATT-CATTATAAAACGAACTAATAATAAA-TAAAA-AA---TTTTT**TA-------GAGTAAA**TTATTCCC-CTCA*
Orthocaulis binsteadii	*AA-----AT**ATCTCATAT-TAT----TTA-AATCTAAT---------AAAATT-CATTATAAAACGAACTAATAATAAA-TAAAA-AA---TTTTT**TA-------GAGTAAA**TTATTCCC-CTCA*
Orthocaulis floerkei	*AG-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTGTGAAACGAACCAATAATAAA-TAAAA-GA---TTTTT**TA-------GAGNAAA**TTATTCTC-CTCA*
Isopaches bicrenatus	*AA-----AT**ATCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTATAAANNNAANNAATAATAAT-NNAAA-AA---TTTTT**TA-------AAGTAAA**TTATTTTTTCTCA*
Obtusifolium obtusum	*AA-----AT**NTCTCATAT-TAT----TTA-AATATAAT---------AAAATT-CATTATAAAACGAATTAATAATAAA----------TTTTT**TA-------AAGGAAA**TTATTCTT-CTCA*
Mylia taylori	*AA-----AT**AATAAAGAATTAT----TTTTAA--TAAC----------TAAAAAA----------GAT-TAATTATAATTTTTTT**AA----------AA**CT-TTC----TCA*
Hattoriella diversiloba	*AACTAAAAA**ATCTAATAT-TAT----TTANAN--TNAT----------AAATT--ATTATA----------TN---NTNNN**TA----------AN**TT-NTCTN--TCA*
Plectocolea obovata	*AACC-GAAT**ATCTAATAT-AAT----CTGTAA--TAAT----------AAATT--ATT--AAAA----------AAA-TA-TTATAGTTTTTTC**TA----------NA**TC-TTATT---CA*
Plectocolea vulcanicola	*AACT-GANT**ATCTNACANAACT----TTATAA--TAAT----------AAATT--AT-ANNNNN----------AAN-TA-TTATAGTNTTTCT**TA----------AT**TT-TTCTT---CA*
Marsupella aquatica	*AATT-AAAT**ATCTAACAC-GAT----TTATAA--TAAA----------AAATT--ATT---AAA----------TAA-TA-TTATAGTTTTTTC**TA----------AA**TT-TTTCC--ACA*
Marsupella sphacellata	*AATA-AAAT**ATTTAGCAC-GAT----TTATAA--TAAT----------AAATT--ATT---AAA----------TAA-TA-TTATAATCTTTTC**TA----------AA**TT-TTCCA---CA*
Marsupella brevissima	*AATT-GANT**ATCTAANAC-GAT----TTATAA--TAAT----------AAATT--ATT---AAA----------AAA-TA-TTATAGTTTCTTN**TA-NNNGTA-----AA**NN-TTTTC---CA*
Gymnomitrium concinnatum	*AATT-GAAT**ATCTAATAC-GAT----TTATAA--TAAT----------AACTT--ATT---AAA----------AAA-TA-TTATAGTTTCTTC**TAtTTCGTA-----AA**TT-TTCTC---CA*

Table 2. Continued.

	188 → 234 →	361 → 407 →
Nardia compressa	*AATT-AAAT**ATCTGACAC-GAT---TTTATAA--TAAT----------CAATT--ATT--AAA----------AAA-TA-TTCCAG--TTT----------AA**TT-TTCTC--GA*
Jamesoniella autumnalis	*AACT-AAAT**ATCTGACAC-GAT---TTTATAA--TAAT----------AAATT--ATT--AAA----------AAA-TA-TTCCAG---T----------AA**TT-TTCTC--GA*
Leiocolea heterocolpos	*AACT-GAAT**ATCCAACAT-AAT---TTTATAA--TAAT----------AAATT--ATNAAANAA----------AAA-TA-TTATAGT-TTT----------TT**TT-TTCTC--TCA*
Leiocolea rutheana	*AACT-AGAT**ATCTAACAT-AAT---TTTATAA--TTAT----------AAATT--ATTAAAAAA----------AAA-TA-TTCTAGT-TTT----------AA**TT-TTCTT--TCA*
Mesoptychia sahlbergii	*AACT-AGAT**ATCTAACAT-AAT---TTTATTA--ACAT----------AAATT--ATTATAAAA----------AAA-TA-TTCTAGT-TTT----------AA**TT-TTCTT--TCA*
Jungermannia pumila	*AACT-AGAT**ATCTAACAN-AA--AAA-TA-TTATAG--TTT----------AA**TT-TTCTT--TCA*
Jungermannia exsertifolia	*AACT-AGAT**ATCTAACATAAAT---TTTATAA--TTAT----------CT**TT-TTCTT--TCA*
Hygrobiella laxifolia	*AATC-AAAT**GTTTAAAAT-GAC---TTTATAA--TTAT----------GAAA----------AAATT-AT----------AA**TT-TTTG---ACA*

decrease of support values at four internal nodes. The topology of the tree where all 48 sequences were included differed from the tree shown in Figure 1 only by combining groups B and C in a low supported (45%) single cluster with *Obtusifolium obtusum* at a basal position.

The MrB tree (not shown) calculated under the assumption of GTR model of sequence evolution with −lnL = 3806.39 score exhibited mean support value of 69%. The MrB tree (not shown) inferred as based on HKY85 model had a log likelihood score −lnL = 3808.61, and its topology differed only slightly from the previous. The differences pertained to the position of *Macrodiplophyllum microdontum* within *Scapania* clade (i.e., one of two clades in the group F), as well as of *Tetralophozia setiformis* and *Crossocalix hellerianus* within the group B. These positions had only low support (31%–45%). The topologies of both 50% majority-rule MrB trees (Fig. 5) were compatible with MPG tree (Figs. 1, 5) with a sole exception—in MrB trees *Saccobasis polita* occupied an outermost position (65%–68% support) in group F.

Neighbor-joining trees based on Kimura model (mean bootstrap value, 68%) and on *p*-distances (mean bootstrap value, 69%) were not distinguishable in their topologies. The NJ tree constructed from Galtier and Gouy distance matrix (not shown) had lower mean bootstrap value (47%) and differed in the position of several branches with those having a low bootstrap support values. Figure 2 shows a 50% majority-rule NJ tree constructed from the *p*-distance matrix (available from authors). The similar A–F groupings as in MPG (Figs. 1, 5) and MrB (Fig. 5) trees were resolved; however, group E but not B branched off first after group A in the NJ tree (Figs. 2, 5).

The parsimony analysis with PAUP* (P-PAUP) of all 48 hepatic sequences yielded 10 equally parsimonious trees with a tree length of 964 steps (CI = 0.54, RI = 0.74). The jackknife 50% majority rule consensus tree (Fig. 3) that included other groups compatible with this tree was only one step longer. The P-PAUP analysis recovered similar groupings (A–F) as the other methods of phylogenetic reconstruction employed herein.

Parsimony analysis with Nona (P-Nona) counting gaps as a fifth nucleotide revealed four well-supported equally parsimonious trees all with a length of 964 steps. The strict consensus of these trees (not shown) was in agreement with the jackknife 50% majority rule consensus tree (not shown) with a sole minor exception—*Plectocolea obovata* branched off as a separate line just below *Hattoriella diversiloba*. Treating gaps as missing data re-

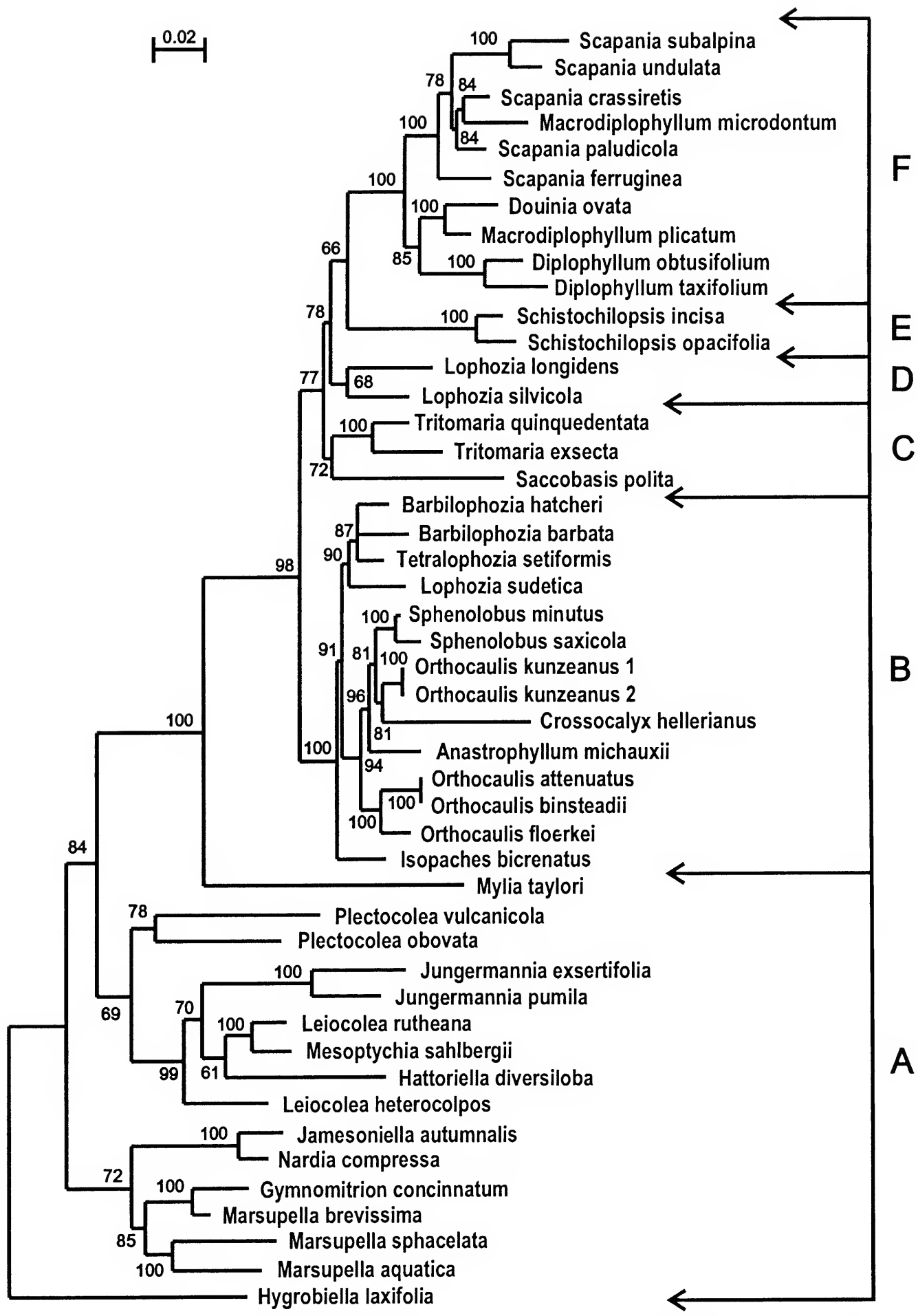

Figure 1. Phylogeny of 46 Jungermanniales species inferred from sequences of the *trn*L-*trn*F intron-spacer region using MetaPIGA metapopulation genetic algorithm. Branch length estimated with ML assuming HKY85 model of sequence evolution (log likelihood score −lnL = 3811.81). MetaPIGA posterior probabilities of clades (branch support values) are indicated near internal nodes, the mean support value is 90%. Scale bar for branch length represents 0.02 expected substitutions per nucleotide site. Six major groups of species was revealed and marked as A–F on the tree.

sulted in 120 equally parsimonious trees. The strict consensus of these trees (Fig. 4) was compatible with the result of Nona analysis with gaps treated as a fifth base, but with a single difference—*Saccobasis polita*, from group C (Fig. 1) or C + D (Figs. 2, 3), combined with group F as in MrB trees (cf. Figs. 4, 5). The reduction of resolution seen in this tree (Fig. 4) indicated the significance of gaps for the results obtained.

The consideration of indel pattern in the align-

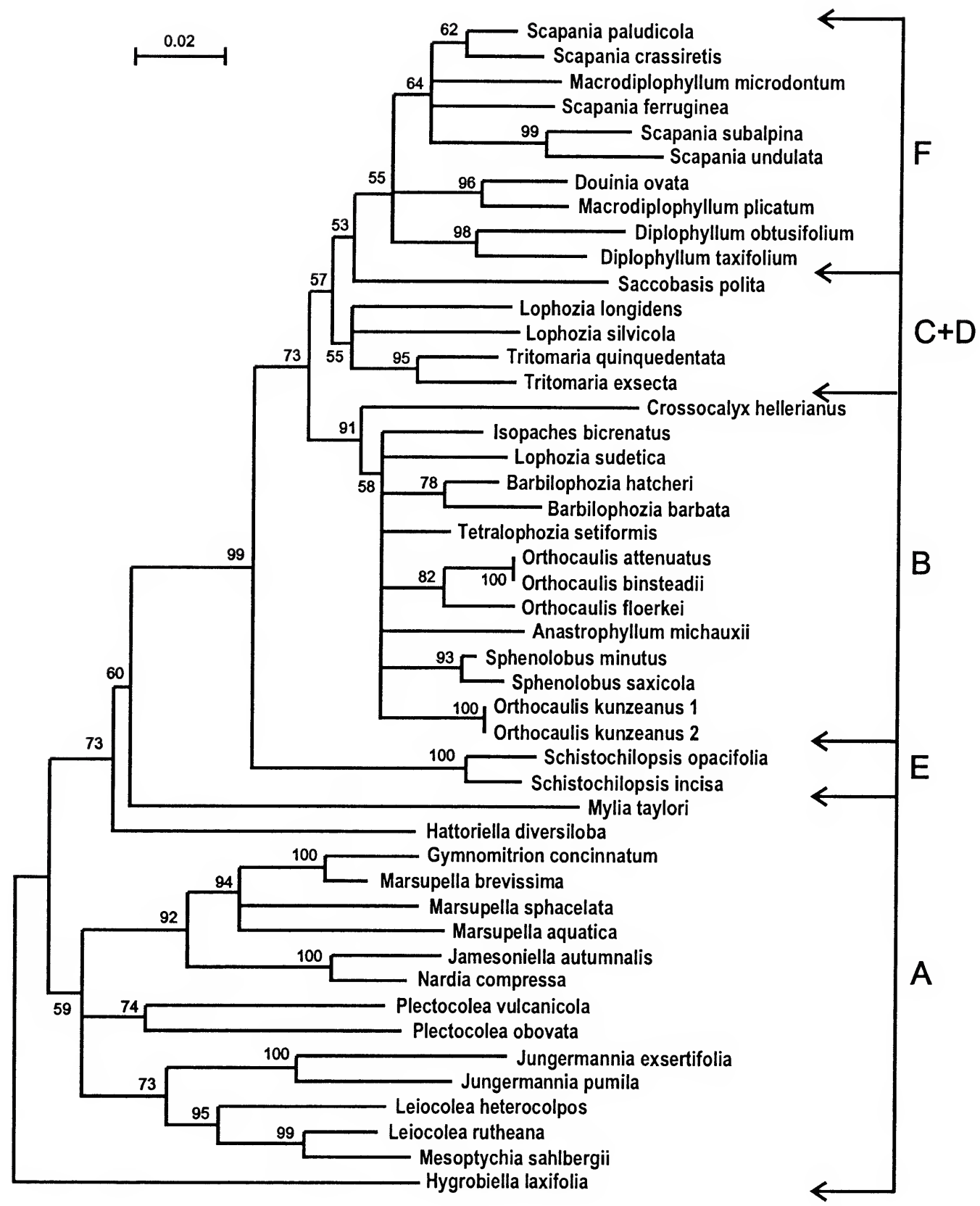

Figure 2. Neighbor-joining 50% majority-rule tree based on uncorrected pairwise sequence divergence (p-distances) inferred from sequences of the *trn*L-*trn*F intron-spacer region of 46 Jungermanniales species. Numbers at internal nodes are bootstrap values based on 1000 replicates. Scale bar for branch length represents 0.02 divergence per nucleotide site. Major groups of species marked as A–F.

ment (Table 2) may yield additional information about relationships of hepatic taxa (discussed below).

Discussion

A wide array of different methods has been developed for phylogenetic inference. They can be roughly divided into those that are based on parsimony, those that are based on use of an explicit model of evolution associated with maximum likelihood estimation, and distance methods based on the minimum evolution paradigm and applied to different models of nucleotide substitution. Numerous papers have been devoted to exploring the epistemology, especially the extent and nature of background knowledge that is accepted in different methods, their statistical properties, and effectiveness at estimating phylogenies over a range of conditions (e.g., Farris, 1983; Hasegawa & Fujiwara, 1993; Stewart, 1993; Hillis et al., 1994; Doyle & Davis, 1998; Lewis, 1998; Nei & Kumar, 2000; Kluge, 2001; Siddall, 2002; de Queiroz & Poe 2001, 2003; Wenzel, 2002; Goloboff, 2003). It is

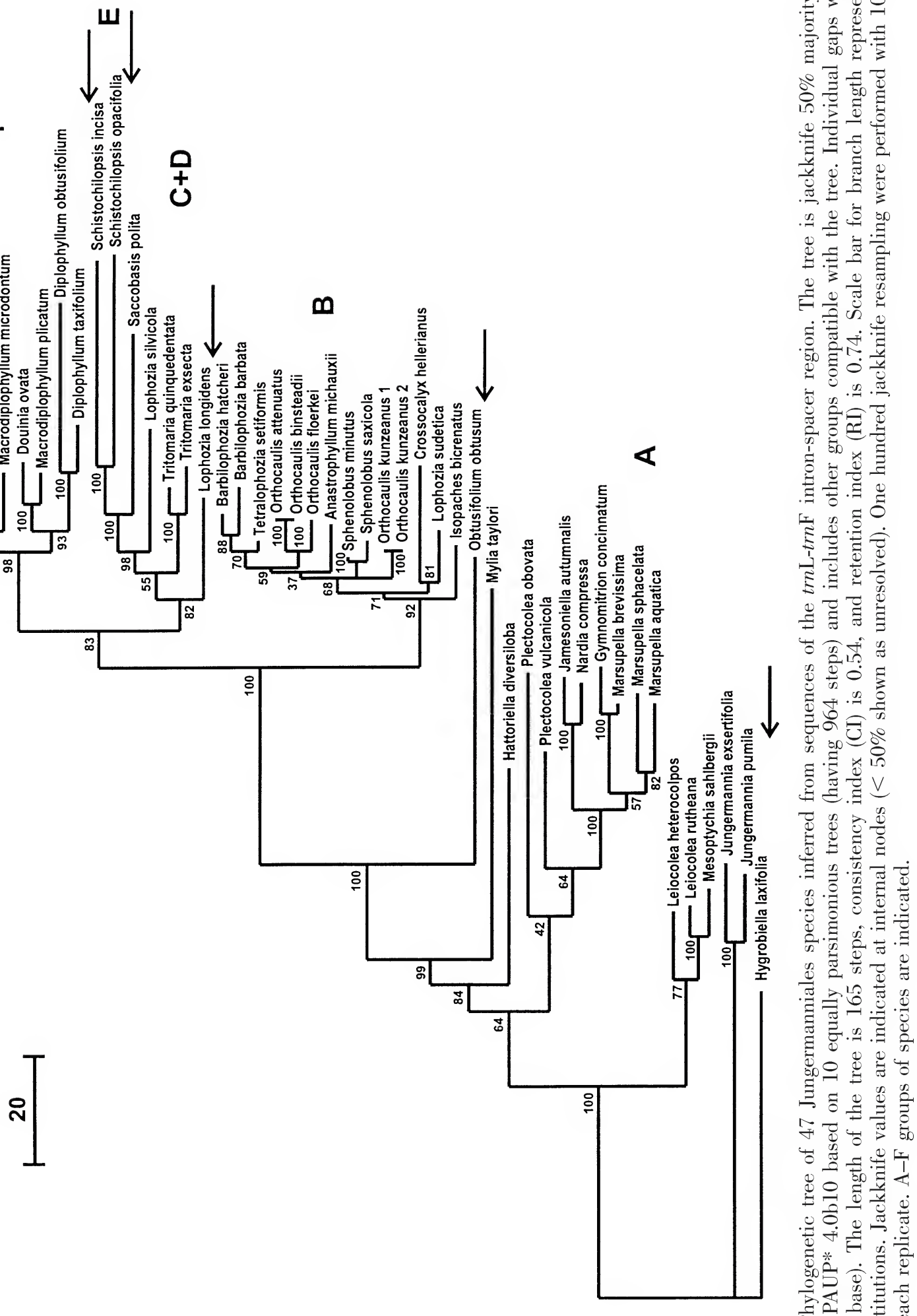

Figure 3. Phylogenetic tree of 47 Jungermaniales species inferred from sequences of the *trn*L-*trn*F intron-spacer region. The tree is jackknife 50% majority rule consensus constructed by PAUP* 4.0b10 based on 10 equally parsimonious trees (having 964 steps) and includes other groups compatible with the tree. Individual gaps were treated as a character (fifth base). The length of the tree is 165 steps, consistency index (CI) is 0.54, and retention index (RI) is 0.74. Scale bar for branch length represents 20 expected nucleotide substitutions. Jackknife values are indicated at internal nodes (< 50% shown as unresolved). One hundred jackknife resampling were performed with 10% of the data to be deleted for each replicate. A–F groups of species are indicated.

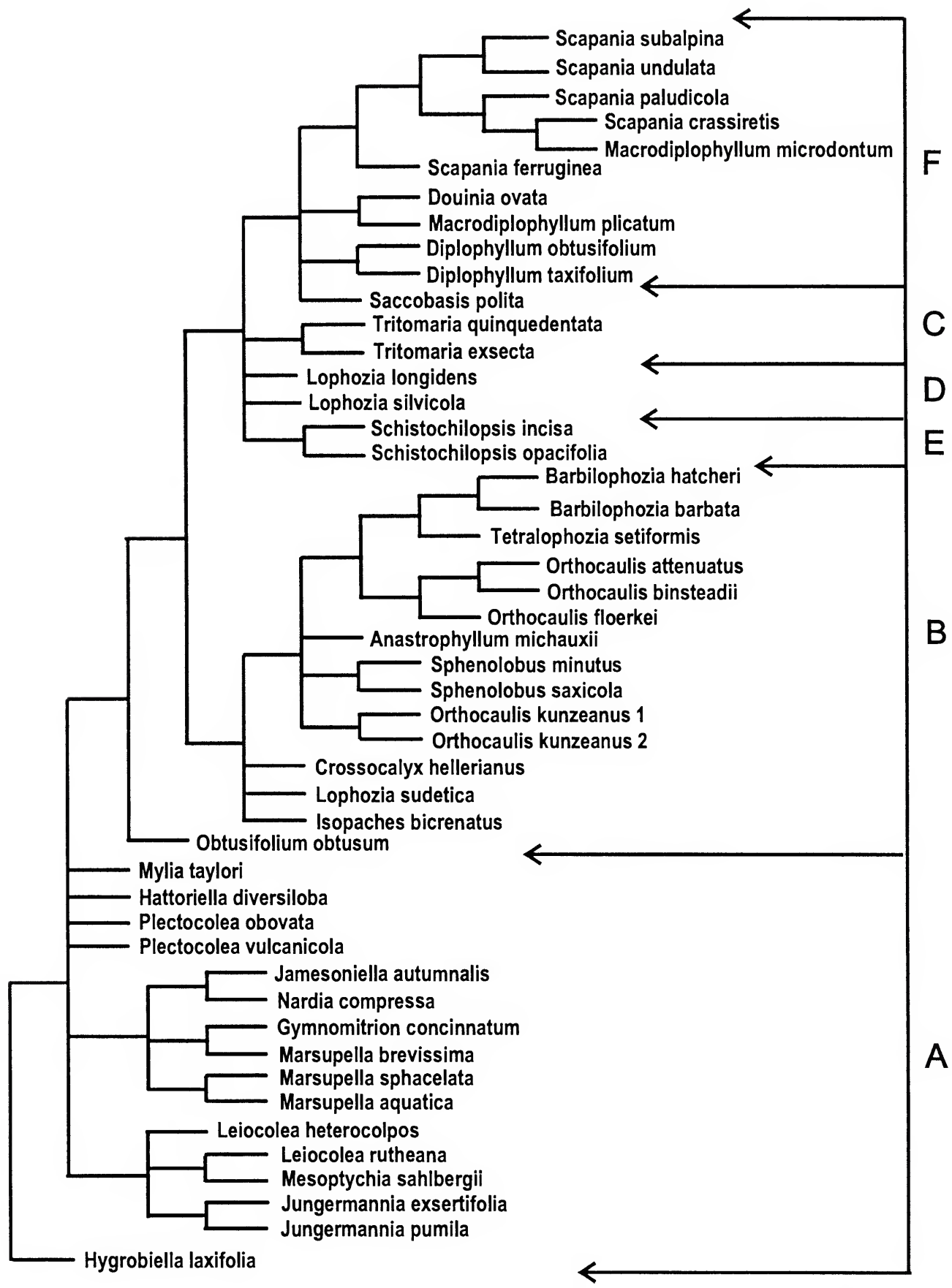

Figure 4. Strict consensus of 120 parsimonious trees of 47 Jungermanniales species inferred from sequences of the *trn*L-*trn*F intron-spacer region. The tree having 964 steps obtained in Nona analyses when gaps were treated as missing data. A–F groups of species are indicated.

evident that no clear consensus exists among phylogeneticists using these different methods.

In this study we performed parsimony based analyses using the algorithms implemented in the programs Nona and PAUP* 4.0b10, two model-based analyses, using maximum likelihood estimation as implemented in the MrB and MPG programs, as well as NJ tree reconstruction based on the minimum evolution principle (e.g., Saitou & Nei, 1987). All these methods yielded quite similar tree topologies (Fig. 5), although they apply different models of nucleotide substitution from the simplest one (*p*-distance) to the model including many variable parameters (GTR). The differences among trees (cf. Fig. 5) include mainly the relative position of internal branches leading to groups B and E. Taxonomic and phylogenetic conclusions that may be inferred from these trees are developed below.

It must be noted, however, that only one gene region was involved in the present study, and our results can hardly be considered as requiring immediate systematic rearrangements. At the same

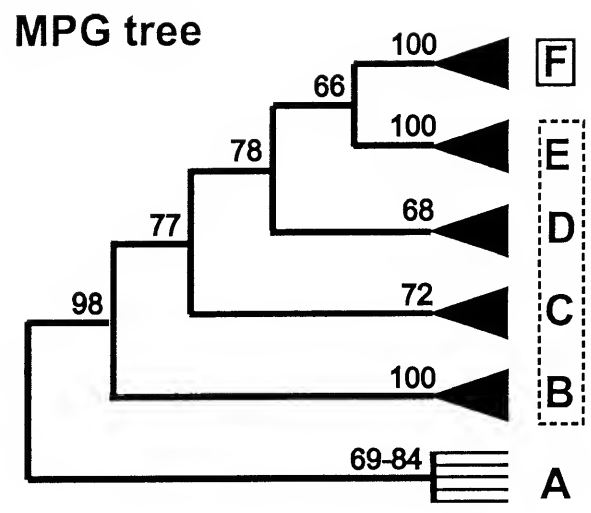

Figure 5. Main features of tree topologies constructed by different methods. Support values in percentages are indicated near internal nodes. Triangles denote monophyletic groups, forks—paraphyletic groups. A–F groups of species are indicated, Scapaniaceae s.l. (F) are in straight line boxes, Lophoziaceae (B, C, E, and F)—in dashed line boxes. In MrB trees the support is given in two values, corresponding to GTR and HKY85 models, respectively.

time, good agreement appears between our results and the phylogenetic analysis of Schill et al. (2004), based on the *trn*L intron and *atp*B-*rbc*L intergenic region sequences for 23 mostly different species from the genera sampled herein as well as three other genera in Jungermanniales suborder Jungermanniinae. This correspondence allows us to discuss possible changes in the systematics of Jungermanniales if our results are confirmed by more thorough sampling of both taxa and characters.

GENERAL CLASSIFICATION OF JUNGERMANNIALES

Our results confirm that in general the traditional subdivision of Jungermanniales has been correct—species belonging to a given family compose clades or sometimes paraphyletic grades: the Gymnomitriaceae and Scapaniaceae are monophyletic, the Lophoziaceae form a paraphyletic group with the Scapaniaceae as a terminal clade (see, however, comments below on positions of *Leiocolea* and *Hattoriella*). The Jungermanniaceae form a paraphyletic basal grade, but only seven species from five genera, three subfamilies were studied, so conclusions about this large family (no fewer than 200 species, 13–20 genera, cf. Schljakov, 1981) must be considered with caution.

FAMILY SCAPANIACEAE (OR SCAPANIACEAE AND DIPLOPHYLLACEAE)

The present analysis resolved the Scapaniaceae in traditional circumscription (cf. Konstantinova et al., 1992; Grolle & Long, 2000, etc.) as a monophyletic group (F) with high support. However, its position as a terminal clade within the Lophoziaceae is fairly unexpected. According to Schuster (1974), Scapaniaceae are apparently related to the less derived Lophoziaceae, but he never stated any immediate affinities between them. More recent treatments keep these families distinct, with the Scapaniaceae considered more distant from Lophoziaceae than Lophoziaceae from Jungermanniaceae (cf. Grolle & Long, 2000; Schuster, 2002).

Within the Scapaniaceae two strongly supported clades (cf. Figs. 1, 3) are found. The first clade includes all five studied species of *Scapania* and one species of *Macrodiplophyllum*. The second clade is composed of *Douinia*, *Diplophyllum*, and one other species of *Macrodiplophyllum*. In general,

this does not contradict Potemkin's (1998) concept, who suggested the segregation of the Diplophyllaceae from the Scapaniaceae. However, another idea by Potemkin (1999), the inclusion of *Macrodiplophyllum* in *Scapania* is supported only partially, as the one species *M. microdontum* was found nested within the *Scapania* clade, whereas *M. plicatum* is resolved within the *Douinia* + *Diplophyllum* clade. Invariably, there was 96%–100% support for this clade formed by *M. plicatum* and *Douinia ovata* in all trees constructed by different methods and assuming different models of evolution. Further examination of these two species is needed to confirm the polyphyly of *Macrodiplophyllum* and explore the possible close relationship of *Macrodiplophyllum plicatum* with *Douinia*.

FAMILY LOPHOZIACEAE AND POSITIONS OF *OBTUSIFOLIUM*, *LEIOCOLEA*, *HATTORIELLA*, AND *MESOPTYCHIA*

All variants of the present analyses resolved most of Lophoziaceae (groups B, C, D, and E) as a paraphyletic grade with the Scapaniaceae s.l. (= Scapaniaceae + Diplophyllaceae, cf. Potemkin, 1998, group F) as a terminal clade (Figs. 1–5).

Obtusifolium was involved only in parsimony analysis (Figs. 3, 4), excluded from the rest by its divergent sequence as explained above (see Results). When included, *Obtusifolium* was found to be a sister to the rest of Lophoziaceae + Scapaniaceae clade (cf. Figs. 3, 4).

Leiocolea and *Hattoriella*, which often are not segregated from the genus *Lophozia* (Grolle, 1983; Crandall-Stotler & Stotler, 2000; Schumacher & Váňa, 2000), were never resolved within the Lophoziaceae; instead they invariably nested in group A. *Leiocolea rutheana* and *Mesoptychia sahlbergii* were found closely related with very high support (99%–100%) in all trees constructed. This fact is unexpected in view of all recent treatments, where *Mesoptychia* was kept in a separate monospecific family Mesoptychiaceae (Steere & Inoue, 1975; Schljakov, 1980; Crandall-Stotler & Stotler, 2000; Grolle & Long, 2000; Schuster, 2002). However, sterile plants of *Mesoptychia* are very similar to the large phenotypes of *Leiocolea rutheana*. Moreover, the immature perianth of *Mesoptychia* is described as beaked; thus, Schuster (1969: 803) stated that "the similarity [of *Mesoptychia*] to *Leiocolea* may prove more than coincidental."

The positions of *Leiocolea heterocolpos* and *Hattoriella diversiloba* in the MPG tree (Fig. 1) and MrB trees (not shown) differ considerably from those in NJ and P trees (Figs. 2–4). In NJ and both P trees (Figs. 2–4) *L. heterocolpos* forms a clade with *L. rutheana* and *Mesoptychia*, but in MPG tree the former is more distantly related to these two species. *Hattoriella diversiloba* in P and NJ trees branches off immediately after *Mylia*, whereas in MPG tree *Hattoriella* (sometimes classified as a subgenus of *Leiocolea*, cf. Schljakov, 1980) forms a clade with *Leiocolea rutheana* + *Mesoptychia*. In any case, the genus *Leiocolea* is not monophyletic and its circumscription has to be reconsidered.

The position of both studied *Leiocolea* species as distant from *Lophozia* is surprising, since many hepaticologists considered *Leiocolea* to be a subgenus of *Lophozia* (cf. Kitagawa, 1965, 1966; Schuster, 1969; Schumacher & Váňa, 2000, etc.). Morphologically, *Leiocolea* differs from *Lophozia* s. str. in many features: it usually has a strongly papillose cuticle, a large ventral segment in most species bearing amphigastria, stems with a homogeneous medula that does not form ventral mycorrizal band, very obliquely inserted leaves, and a peculiar perianth shape, and all species except *L. heterocolpos* lack gemmae. Also, most species of *Leiocolea* are more or less calciphilous, whereas most of *Lophozia* s. str., as well as most of Lophoziaceae, are acidophilous (see e.g., Schuster, 1969).

The result of the present analysis can be interpreted in favor of the placement of *Leiocolea* and *Hattoriella* outside the Lophoziaceae (perhaps in Mesoptychiaceae). Besides the tree topologies, this is supported by the characteristic indels in *trn*L-F region. As it is outlined in Table 2, Lophoziaceae and Scapaniaceae differ markedly from Jungermanniaceae, Gymnomitriaceae, and Mesoptychiaceae, and *Leiocolea* and *Hattoriella* fit the group of three latter families.

THE GENERAL CLASSIFICATION OF LOPHOZIACEAE (EXCLUDING *LEIOCOLEA* AND *HATTORIELLA*)

Most species of Lophoziaceae included in the present study form a paraphyletic group (B, C, D, E).

The genus *Lophozia* is represented in our analysis by three species. Two of them, *L. sylvicola* and *L. longidens* (group D), belong to *Lophozia* sect. *Lophozia*. The third one, *L. sudetica*, which was sometimes classified in a separate section *Lophozia* sect. *Sudeticae* Schljakov (cf. Grolle & Long, 2000) but invariably within this genus, appears in group B, quite unrelated to two species of *Lophozia* sect. *Lophozia*. At the same time, both latter species form a clade in MPG (Fig. 1, group D), MrB, and NJ (Fig. 2) trees (in the latter case with low bootstrap support, 33%, not shown in Fig. 2), while P-PAUP

analysis (Fig. 3) resolves them as not monophyletic, but nested in the broader clade, which includes also *Schistochilopsis*, *Tritomaria*, and *Saccobasis* (groups C + D + E). In the MPG and MrB trees the three latter genera and *Lophozia* sect. *Lophozia* form a basal grade (C + D + E) leading to the Scapaniaceae s.l. (group F).

This topology challenges the family level classification: either the Scapaniaceae should be lumped with the Lophoziaceae, or the Lophoziaceae should be much reduced, with clades B, C, D, E being recognized as separate families.

LOPHOZIACEAE S. STR. (GROUPS C + D + E)

Results of the MPG (Fig. 1) analysis do not contradict both the treatment of *Saccobasis* as a subgenus of *Tritomaria* (Schuster, 1969; Paton, 1999; Grolle & Long, 2000, etc.), or its acceptance as a separate genus (Buch, 1936; Schljakov, 1980). However MrB, NJ, and P tree topologies (Figs. 2–4) support the latter recognition, as *Saccobasis* was not a sister to either *Tritomaria* species included there.

According to all tree topologies (Figs. 1–4) as well as by its characteristic 41-nucleotide-long deletion in positions 236–276 (Table 2), *Schistochilopsis* (group E) is confirmed as a separate genus, unless one would like to lump *Lophozia*, *Tritomaria*, *Saccobasis*, and *Schistochilopsis* in a single genus. Morphologically, *Schistochilopsis* is well differentiated from *Lophozia* s. str. in having malleable 2- or 3(to 4)-lobed leaves, that are usually 2- to 4(to 5)-stratose at the base and sometimes up to mid-leaf (this feature seems unique in Lophoziaceae); succulent stems; a not distinctly dorsally differentiated medula of stem and only sometimes with 1 or 2 rows of smaller cells ventrally; a very large ventral segment of stem of 6 to 12 and sometimes even more cells, also bearing no amphigastria; and in most species oil bodies are small (2–4 μm) and numerous (25–40[–60] per cell).

LOPHOZIACEAE OTHER THAN THE CORE OF THE FAMILY (GROUP B)

Group B forms a clade with high support, 91%–100%, in all analyses (cf. Figs. 1–3, 5). This clade includes species that were classified in *Tetralophozia*, *Barbilophozia*, *Orthocaulis*, *Anastrophyllum*, *Sphenolobus*, *Crossocalyx*, and *Isopaches*, as well as *Lophozia sudetica*. Though the latter species has been considered in a distinct section of *Lophozia* (cf. Schljakov, 1980; Grolle & Long, 2000), its quite remote position from the rest of *Lophozia* s. str. in our analysis is surprising and needs further confirmation.

Using the broad generic concept of Schuster (1969), clade B includes representatives of only three genera—*Chandonanthus* (including *Tetralophozia*), *Lophozia* (including *Orthocaulis*, *Barbilophozia*, *Isopaches*, *Lophozia sudetica*), and *Anastrophyllum* (including *Anastrophyllum*, *Sphenolobus*, *Crossocalyx*). The topology of the MPG, MrB, and parsimony trees (Fig. 1), however, strongly contradicts the acceptance of two latter genera in such a broad sense. Representatives of *Anastrophyllum* s.l. nest within *Lophozia* s.l., and, moreover, one species of *Lophozia* s.l. (*Orthocaulis kunzeanus*) nests within *Anastrophyllum* s.l. (cf. Figs. 1, 3).

We were surprised to find *Orthocaulis kunzeanus* among *Anastrophyllum* s.l. and checked this result by sequencing the region under investigation from another specimen from a different locality. Both sequences, however, were found to be identical. Thus, it is quite likely that *Orthocaulis kunzeanus* is not congeneric with other species sampled from this genus, namely, *Orthocaulis floerkei*, *O. attenuatus*, and *O. binsteadtii* (Figs. 1–4). *Orthocaulis kunzeanus* has been always considered as belonging to the same genus as the above three species. Within *Orthocaulis*, it was often assumed to be among the more primitive species (Schuster, 1951; Potemkin, 1999) and the only species of *Orthocaulis* having 2-lobed leaves rather than the 3- or 4-lobed leaves characteristic of the rest of the genus. In this respect *O. kunzeanus* resembles the species of *Anastrophyllum* s.l. The similarity of *Anastrophyllum* s.l. and *Orthocaulis kunzeanus* in the leaf shape was also outlined by Schuster (1969), who considered this similarity to be superficial.

Tetralophozia was found in our analysis rather close to *Barbilophozia* (Figs. 1, 3, 4). This relationship is in disagreement with the systematic concept of many recent authors who place *Tetralophozia* in a separate subfamily, Chandonanthoideae H. Inoue (cf. Schljakov, 1980; Schuster, 2002), but is congruent with treatments by other authors (e.g., Konstantinova et al., 1992; Grolle & Long, 2000) that this genus belong to the subfamily Lophozioideae.

According to obtained topologies for Lophoziaceae (Figs. 1–4), many genera in their broad concepts (Kitagawa, 1965, 1966; Schuster, 1969; Grolle & Long, 2000) are not monophyletic. Our results are strongly in favor of narrowly defined and, hence, more homogeneous genera (cf. Buch, 1933, 1936; Arnell, 1956; Schljakov, 1980, 1981; Konstantinova et al., 1992).

JUNGERMANNIACEAE, GYMNOMITRIACEAE, AND *LEIOCOLEA*, *HATTORIELLA*, AND *MESOPTYCHIA* (GROUP A)

The positions of *Leiocolea*, *Hattoriella*, and *Mesoptychia* on topologies (Figs. 1–3) were discussed above.

Mylia was found as a sister group to the Lophoziaceae + Scapaniaceae clade (B–F groups) in all trees (Figs. 1–4). The position of *Mylia* as an intermediate branch between Lophoziaceae and Jungermanniaceae is in rather good agreement with the traditional understanding of its place in the classification: more commonly as a separate subfamily of the Jungermanniaceae (Grolle & Long, 2000).

Plectocolea and *Jungermannia* were resolved as quite distinct from each other, which supports their generic independence (Schljakov, 1981), though until recently *Plectocolea* was treated by some authors as a subgenus of *Jungermannia* (Grolle & Long, 2000) or *Solenostoma* (Schuster, 1969, 2002).

Nardia compressa and *Jamesoniella autumnalis* form a clade with support values of 100% in all analyses. This is rather unexpected as Schljakov (1981) classified these two genera in different subfamilies, and Grolle and Long (2000) in different families. Furthermore, these two species form a sister group to the Gymnomitriaceae (*Gymnomitrion concinnatum*, *Marsupella brevissima*, *M. sphacelata*, and *M. aquatica*), far from both *Jungermannia* and *Plectocolea*, with 92%–100% support in NJ (Fig. 2), MrB (not shown), and P-PAUP (Fig. 3) trees, and 72% support in MPG (Fig. 1) analysis. The relationship of the Gymnomitriaceae, *Nardia*, and *Jamesoniella* is confirmed by a synapomorphic deletion (3 nucleotides) in positions 284–287 of the alignment (Table 2). Close relationship of *Marsupella* and *Nardia* was never suggested before, but it was found also by Schill et al. (2004) in a result of *atp*B-*rbc*L intergenic region analyses.

Gymnomitriaceae is resolved as monophyletic in all analyses, either with high (Figs. 1, 2) or low (Fig. 3) support. *Marsupella aquatica* was found closely related to *M. sphacellata*; both species belong to the section *Marsupella*. At the same time *M. brevissima* appeared closer to *Gymnomitrion concinnatum* than to two former *Marsupella* species, and the presence of common insertion (six nucleotides) after position 362 (Table 2) confirm their affinity. This fact is not very surprising, because the boundaries between these two genera have been ill defined (Müller, 1951–1958; Schuster, 1974, etc.). *Marsupella brevissima* belongs to the subgenus *Homocraspis* (Schiffn.) Grolle, which is characterized by a perianth that is highly reduced to absent, as it is in most *Gymnomitrion* species, while in *Marsupella* sect. *Marsupella* perianth is always well developed.

The present analysis can be considered as an exercise to explore whether the highly variable noncoding regions of chloroplast DNA, the *trn*L-F intron and spacer, include information that can be used to assess phylogeny of Lophoziaceae. This proved to be true, but, in order to make robust estimations about phylogeny, our sampling must be greatly expanded (both in terms of characters and taxa). Future analyses should utilize methods such as direct optimization (Wheeler, 1996) that allow the inclusion of entire sequences that show length variation. It was evident that alignment gaps affected our results to a great extent as illustrated by the fact that resolution was much reduced when these gaps were treated as missing data. Therefore, it is extremely important that when they are included in the future analyses they are treated as objectively as possible.

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Appendix 1. Specimen list with DDBJ/EMBL/GenBank accession numbers. For *Orthocaulis kunzeanus* only one sequence has been submitted to GenBank, as specimens (1) and (2) were found to be identical.

Taxon	Herbarium voucher	Accession no.
Lophoziaceae		
Anastrophyllum michauxii (F. Weber) H. Buch	Russia, Buryatiya, 7 Aug. 1999, *Bakalin s.n.* (KPABG)	AY327763
Barbilophozia barbata (Schmidel ex Schreb.) Loeske	Russia, Buryatiya, *Konstantinova 951/1-01* (KPABG)	AF519190
Barbilophozia hatcheri (A. Evans) Loeske	Russia, Murmansk Prov., *Konstantinova 100-94* (KPABG)	AY327785
Crossocalyx hellerianus (Nees ex Lindenb.) Meyl.	Russia, Kareliya, 6 Aug. 1997, *Bakalin s.n.* (KPABG)	AY327780
Hattoriella diversiloba (S. Hatt.) Inoue	Russia, Buryatiya, 9 Aug. 1999, *Bakalin s.n.* (KPABG)	AY327772
Isopaches bicrenatus (Schmidel ex Hoffm.) H. Buch	Russia, Yakutiya, 18 Jul. 2000, *Bakalin s.n.* (KPABG)	AY327788
Leiocolea heterocolpos (Thed. ex Hartm.) H. Buch	Russia, Kareliya, 17 Aug. 2001, *Bakalin s.n.* (KPABG)	AY327776
Leiocolea rutheana (Limpr.) Müll. Frib.	Russia, Murmansk Prov., *Konstantinova 37-94* (KPABG)	AF519186
Lophozia longidens (Lindb.) Macoun	Russia, Kareliya, 22 Jul. 1997, *Bakalin s.n.* (KPABG)	AF519185
Lophozia silvicola H. Buch	Russia, Kareliya, 2 Aug. 1998, *Bakalin s.n.* (KPABG)	AF519197
Lophozia sudetica (Nees) Grolle	Russia, Murmansk Prov., 4 Jun. 1998, *Bakalin s.n.* (KPABG)	AF519195
Obtusifolium obtusum (Lindb.) S. W. Arnell	Russia, Murmansk Prov., 1 Jul. 2001, *Bakalin s.n.* (KPABG)	AY327769
Orthocaulis attenuatus (Mart.) A. Evans	Russia, Buryatiya, 3 Aug. 1999, *Bakalin s.n.* (KPABG)	AY327765
Orthocaulis binsteadii (Kaal.) H. Buch	Russia, Murmansk Prov., 8 Sep. 1997, *Konstantinova s.n.* (KPABG)	AY327764
Orthocaulis floerkei (F. Weber & D. Mohr) H. Buch	Russia, Buryatiya, *Konstantinova 150/2-01* (KPABG)	AF519191
Orthocaulis kunzeanus (Huebener) H. Buch (1)	Russia, Murmansk Prov., *Konstantinova 181-02* (KPABG)	AF519192
Orthocaulis kunzeanus (Huebener) H. Buch (2)	Russia, Kareliya, 24 May 2000, *Bakalin s.n.* (KPABG)	AF519192
Saccobasis polita (Nees) H. Buch	Russia, Murmansk Prov., 1 Jul. 2001, *Bakalin s.n.* (KPABG)	AF519194
Schistochilopsis incisa (Schrad.) Konstantinova	Russia, Caucasus, 23 Aug. 1999, *Korotkov s.n.* (MW)	AY327784
Schistochilopsis opacifolia (Meyl.) Konstantinova	Russia, Murmansk Prov., *Konstantinova 180-02* (KPABG)	AY327781
Sphenolobus minutus (Schreb.) Berggr.	Russia, Kareliya, 24 Jul. 1998, *Bakalin s.n.* (KPABG)	AY327766
Sphenolobus saxicola (Schrad.) Steph.	Russia, Buryatiya, *Konstantinova 146/11-01* (KPABG)	AY327767
Tetralophozia setiformis (Ehrh.) Schljakov	Russia, Murmansk Prov., *Konstantinova 145/3-89* (KPABG)	AY327787
Tritomaria exsecta (Schmidel) Loeske	Russia, Amurskaya Prov., 14 Jul. 2000, *Bakalin s.n.* (KPABG)	AY327768
Tritomaria quinquedentata (Huds.) H. Buch	Russia, Kareliya, 2 Jul. 1997, *Bakalin s.n.* (KPABG)	AY327786
Cepaloziaceae		
Hygrobiella laxifolia (Hook.) Spruce	Russia, Murmansk Prov., *Konstantinova 29/1-94* (KPABG)	AF519187
Diplophyllaceae		
Diplophyllum obtusifolium (Hook). Dumort.	Russia, Amurskaya Prov., 14 Jul. 2000, *Bakalin s.n.* (KPABG)	AY327782
Diplophyllum taxifolium (Wahlenb.) Dumort.	Russia, Kareliya, 28 Jul. 1998, *Bakalin s.n.* (KPABG)	AY327762
Douinia ovata (Dicks.) H. Buch	U.S.A., Washington, *Potemkin 95/401* (LE)	AY327778

Appendix 1. Continued.

Taxon	Herbarium voucher	Accession no.
Gymnomitriaceae		
Gymnomitrion concinnatum (Lightf.) Corda	Russia, Murmansk Prov., *Konstantinova 366-00* (KPABG)	AF519202
Marsupella aquatica (Lindenb.) Schiffn.	Russia, Murmansk Prov., *Konstantinova 152/5-87* (KPABG)	AF519201
Marsupella brevissima (Dumort.) Grolle	Russia, Murmansk Prov., *Konstantinova 28-94* (KPABG)	AY327774
Marsupella sphacelata (Gieseke ex Lindenb.) Dumort.	Russia, Kemerovskaya Prov., *Konstantinova 65/1-00* (KPABG)	AF519200
Jungermanniaceae		
Jamesoniella autumnalis (DC.) Steph.	Russia, Kareliya, 6 Aug. 1997, *Bakalin s.n.* (KPABG)	AY327773
Jungermannia exsertifolia Steph.	U.S.A., Wyoming, *Konstantinova A69/5-95* (KPABG)	AY327775
Jungermannia pumila With.	Russia, Tyva, 10 Jul. 1999, *Bakalin s.n.* (KPABG)	AY327771
Mylia taylori (Hook.) Gray	Russia, Buryatiya, 26 Jul. 1990, *Kazanovskiy s.n.* (LE)	AY327779
Nardia compressa (Hook.) Gray	Canada British Columbia, *Konstantinova A97/1-95* (KPABG)	AF519188
Plectocolea obovata (Nees) Lindenb.	Russia, Murmansk Prov., *Konstantinova 182-02* (KPABG)	AY327789
Plectocolea vulcanicola Schiffn.	Russia, Kamchatskaya Prov., 29 Aug. 2001, *Bakalin s.n.* (KPABG)	AY327777
Mesoptychiaceae		
Mesoptychia sahlbergii (Lindb.) A. Evans	Russia, Chukotka, 10 Aug. 1979, *Afonia s.n.* (KPABG)	AF519189
Scapaniaceae		
Macrodiplophyllum microdontum (Mitt.) Perss.	Russia, Buryatiya, *Konstantinova 146/12-01* (KPABG)	AF519199
Macrodiplophyllum plicatum (Lindb.) Perss.	Russia, Kamchatskaya Prov., 22 Aug. 200-1, *Bakalin s.n.* (KPABG)	AF519198
Scapania crassiretis Bryhn	Russia, Murmansk Prov., *Konstantinova 363/2-00* (KPABG)	AY327761
Scapania ferruginea (Lehm. & Lindenb.) Gottsche, Lindenb. & Nees	India, Sikkim, *Long 22492* (LE)	AF519193
Scapania paludicola Loeske & Müll. Frib.	Russia, Kareliya, 11 Aug. 1997, *Bakalin s.n.* (KPABG)	AF519196
Scapania subalpina (Nees ex Lindenb.) Dumort.	Russia, Murmansk Prov., *Konstantinova 361/1-00* (KPABG)	AY327783
Scapania undulata (L.) Dumort.	Russia, Murmansk Prov., *Konstantinova 344/1* (KPABG)	AY327760

MOLECULAR PHYLOGENY OF THE LIVERWORT ORDER PORELLALES (MARCHANTIOPHYTA, JUNGERMANNIOPSIDA)[1]

Inkeri Ahonen[2]

Abstract

The phylogeny of Porellales and Radulales was studied, with special emphasis on the relationships within the liverwort family Lejeuneaceae. The phylogenetic analysis was based on molecular markers from three genic regions: (1) the internal transcribed spacer 2 (ITS2) sequence between the nuclear genes encoding the 26S and 5.8S ribosomal RNA; (2) partial sequences of the chloroplast genes *trn*L and *trn*F including the spacer between them; and (3) the partial sequence of the chloroplast gene *rbc*L. The parsimony analyses were performed using the optimization alignment procedure. Optimization alignment was found to be an efficient way to find the hidden phylogenetic signal within highly variable regions in simultaneous analysis. At the same time, optimization alignment was found to be susceptible to long branch attraction and repulsion caused by independent indel events in unrelated nucleotide sequences. The fixed states method also showed this same tendency. Variable regions had a positive effect on the phylogenetic structure, and prevented the long branch attraction. It was evident that transformation and gap costs used in the parsimony analysis strongly affect the results, and the optimal parameter values have to be explored for each data set individually. The Radulaceae and *Ptilidium pulcherrimum* were resolved inside the Porellales. The results clearly indicated that *Jubula* and *Frullania* belong to separate families, Jubulaceae and Frullaniaceae, and that the family Lejeuneaceae is monophyletic, except for *Nipponolejeunea,* which belongs to the Jubulaceae. Inside the Lejeuneaceae two monophyletic subfamilies, the Ptychanthoideae and Lejeuneoideae, could be recognized, those being sister clades in the tree with best incongruence length difference (ILD) value. The Cololejeuneae could not be assigned a tribal status. *Odontolejeunea lunulata* of the Brachiolejeuneae was basal in the Lejeuneaceae and sister to the Ptychanthoideae–Lejeuneoideae clade.

Key words: Frullaniaceae, Hepaticae, ITS2, Jubulaceae, Jungermanniopsida, Lejeuneaceae, long branch attraction, long branch repulsion, Marchantiopsida, optimization alignment, parsimony, phylogeny, Porellaceae, Radulaceae, *rbc*L, sensitivity analysis, *trn*F, *trn*L.

The liverwort suborder Porellineae R. M. Schust. was established by Schuster (1953). It was the largest of the eight suborders of the Jungermanniales R. M. Schust. (Schuster, 1953, 1958, 1963a), comprising more than half of the leafy liverwort species. According to him, the Porellineae marked the culmination of liverwort evolution in their cytology, morphology, and ecology (Schuster, 1980a). There was a general tendency for reduction and simplification in Porellineae morphology and life cycle, and most members of this suborder are epiphytes on angiosperm bark and leaves. He considered this to be indicative of their recent coevolution with angiosperms.

The Porellineae comprise the Porellaceae Cavers, Goebeliellaceae Verd., Lejeuneaceae, and Frullaniaceae Lorch. At first, Schuster also included the family Radulaceae (Dumort.) K. Müll. (Schuster, 1953), but later removed it from Porellineae to a suborder of its own, the Radulineae R. M. Schust. (Schuster, 1958). The diagnostic features that supported the monophyly of the Porellineae according to Schuster (1953) are many: a similar ecology, two-lobed leaf, the ventral lobe forming a water-sac, asexual reproduction by many-celled discoid brood-bodies or caducous leaves, restriction of rhizoids to the underleaf bases, a reduced sporophyte with short seta, and, at least according to Schuster, a perianth that is never broad apically (Schuster, 1958). In the Radulaceae, however, the rhizoids are restricted to the lobule, underleaves are completely lacking, and the capsule is dorsi-

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ventrally compressed and wide at the mouth. Schuster (1963a) later added that in the Porellineae the leaf is basically trifid and compound and bifid in the Radulineae. Interestingly, Schuster himself admitted (1980a) that even the typic family Porellaceae fails to show the trifid leaf structure. In addition, Mizutani (1961) considered the perianth of the Porellaceae to be wide at the mouth.

Schuster (1980a) suggested that the Porellineae-type leaf might have developed by reduction from the trifid leaf, like that of *Ptilidium* Nees. Other proof of the common ancestry of the Porellinae and *Ptilidium* would be the shared *Frullania*-type branching. However, *Frullania*-type branching is also found in several other families, e.g., in Lepidolaenaceae.

The suborder Porellineae was elevated to the order Porellales (R. M. Schust.) Schljakov emend. Stotler & Stotl.-Crand. by Schljakov (1972) and Crandall-Stotler and Stotler (2000). Schljakov (1972) divided his superorder Jungermannianae Schljakov into four orders. He distinguished the Porellales among others by the two-lobed leaves, the anterior lobe being clearly smaller, and the rhizoids that are confined either to the base of the underleaves, or to the leaf lobules. The Porellales contained two suborders: the Radulineae, with a single family Radulaceae Cavers, and Porellineae with the Porellaceae, Jubulaceae Limpr., Lejeuneaceae Cavers emend. Verd. and Goebeliellaceae. The suborder Ptilidiineae R. M. Schust. belonged to the order Ptilidiales Schljakov.

Crandall-Stotler and Stotler (2000) based their classification on a cladistic analysis of 61 morphological characters. Consequently, they divided the phylum Marchantiophyta (Kenrick & Crane, 1997) into two classes, the Marchantiopsida and the Jungermanniopsida. They further divided the Jungermanniopsida into two subclasses, Metzgeriidae and Jungermanniidae, and Jungermanniidae into five orders; among them are the Porellales and Radulales. It is worth noting that there were no representatives of the order Radulales (R. M. Schust.) Stotler & Stotl.-Crand. in their cladistic analysis.

The Porellales (R. M. Schust.) Schljakov emend. Stotler & Stotl.-Crand. contained two suborders, Porellineae R. M. Schust. and Jubulineae (Spruce) Müll. Frib., with the Goebeliellaceae, Jubulaceae, Bryopteridaceae Stotler, and Lejeuneaceae composing the Jubulineae. The Porellineae comprised only the Porellaceae, which according to Crandall-Stotler and Stotler (2000), differs from the Jubulineae in many respects. Leaves in Porellineae are fundamentally two parted, compared to three parted in the Jubulineae. The leaves of the Porellineae never form a water-sac (a sac-like structure formed by the folding and unequal growth of the ventral leaf lobe), and their perianths are without a beak-like outgrowth on the upper end. Their sporophyte is enclosed by a well-developed shoot calyptra, whereas a true calyptra is formed by the Jubulineae. Furthermore, the capsule wall of the Porellineae is multistratose, compared to bistratose in the Jubulineae, and their elaters are free, unlike those of the Jubilineae, which are attached to valve apices.

The Frullaniaceae and Lejeuneaceae have been regarded as monophyletic entities even in some earlier studies, at the subordinal (Buch, 1934; Arnell, 1956) or tribal (Müller, 1954–1957) level. In those studies the Porellaceae, Radulaceae, and Ptilidiaceae were placed in the suborder Jungermaniineae Spruce.

This study is the first attempt to use molecular characters to clarify the taxonomic relationships of the liverwort order Porellales. It is a preliminary study with a tentative selection of taxa. Unfortunately, fresh material of the monotypic family Goebeliellaceae was not available. Three genomic regions, the nuclear ITS2, partial sequences of the *rbc*L gene, and the *trn*L-*trn*F regions from the chloroplast, were sampled to test the monophyly of the Porellales and to compare former classifications proposed by Schuster (1953, 1958, 1963a), Schljakov (1972), and Crandall-Stotler and Stotler (2000), especially concerning the position of the family Radulaceae. Another aim of this study was to test whether the molecular data would support the assignment of the genera *Frullania* and *Jubula* to one family or to two separate families.

Special emphasis is given here to the family Lejeuneaceae Cas.-Gil., the largest extant liverwort family, which embraces from 1/8 to 1/6 of all the liverwort species (there are about 1400 species of Lejeuneaceae and, according to Crandall-Stotler and Stotler (2000), there are from 6000 to 8000 species of liverworts). The generic, tribal, and subfamily boundaries in this huge family of ca. 1000 species are in many cases unclear, probably due to their rapid and recent evolution. Many different divisions of this family have been proposed (e.g., Spruce, 1884; Herzog, 1957; Mizutani, 1961, 1985; Schuster, 1963b; Gradstein, 1994, 2001; Weis, 2001; Ahonen, 2003; Gradstein et al., 2003). The most widely accepted of these subfamilies are Ptychanthoideae Mizut., Lejeuneoideae, and Cololejeuneoideae Herzog (tribe Cololejeuneae, Gradstein, 2001). Phylogenetic inferences based on molecular data resolved the Ptychanthoideae and the Lejeuneoideae as sister groups (Ahonen et al., 2003), whereas cladistic analyses based on mor-

phological characters resulted in a paraphyletic Ptychantoideae (Weis, 2001; Gradstein et al., 2003). The Cololejeuneoideae has been resolved as a member of the Lejeuneoideae in all these studies, but only in Ahonen et al. (2003) could it have been assigned a tribal status without violating the monophyly of other tribes. One purpose of this study was to confirm the relationships of these subfamilies. In addition, the relationships of tribe Cololejeuneae Gradst. to the Tuyamaelloideae R. M. Schust. was tested. The Tuyamaelloideae is one of the several small subfamilies that Schuster (1963b) described. Gradstein (1994) and Mizutani (1985) merged it with Lejeuneoideae. The close relationships of the Cololejeuneae and Tuyamaelloideae have been pointed out by Mizutani (1961) and Schuster (1980a), and these clades formed a single monophyletic group based on morphological characters in Weis (2001).

The Families Included in the Study

PORELLACEAE

This family includes about 100 species, with great plasticity within species in their morphology (Schuster, 1980a). The family has been divided into the genera *Porella* (Dill.) L., *Ascidiota* Mass. (Steere & Schuster, 1960), and *Macvicaria* Nichols. (Hattori, 1978). Schuster (1980a) accepted only *Porella* and *Ascidiota,* whereas Crandall-Stotler and Stotler (2000) accepted all the three genera. The genus *Porella* includes almost all the species of the family, *Ascidiota* and *Macvicaria* both being monotypic. The Porellaceae grow often as epiphytes on wood or on rock.

FRULLANIACEAE AND JUBULACEAE

There has been controversy whether the Frullaniaceae Lorch and Jubulaceae Klinggr. ex Massal. should be treated as only one family. Guerke (1978) used the family name Jubulaceae, with two subfamilies Jubuloideae and Frullanioideae. In the Jubuloideae, he included *Jubula* Dumort., and in Frullanioideae *Amphijubula* R. M. Schust., *Frullania* Raddi, *Neohattoria* Kamimura, *Schusterella* S. Hatt. et al., and *Steerea* S. Hatt. & Kamim.

Asakawa et al. (1979) proposed that there should be two families, the Frullaniaceae and Jubulaceae. They based their view on chemical and morphological differences of *Jubula* and *Frullania,* such as the structure of the seta, germination of spores, the structure of the capsule wall, number and type of the subfloral innovations, the type of androecial branches, presence of bracteoles, the shape of the initial leaves of branches, insertion of the leaf-lobe to the stem, presence of secondary pigmentation, and pilose teeth in leaves. They included the genera *Frullania, Schusterella,* and *Steerea* in the Frullaniaceae, and *Jubula* and *Neohattoria* in the Jubulaceae. Hattori (1982) presented ample evidence from the morphology of vegetative parts, sporophytes, and sexual organs as well as chemical properties and chromosomes in favor of recognizing two different families. Hattori and Mizutani (1982) added what they considered as one of the most important reasons for separation: in *Jubula* four small, pigmented areas composed of small, thick-walled cells differentiate at the capsule base, along which the capsule dehisces into four valves, whereas in *Frullania* there are two such areas opposite to each other. Yuzawa (1991) also recognized the above-mentioned characters that distinguish *Jubula* and *Frullania* and added one more, the presence of two to four, or even more, archegonia per gynoecium in *Frullania,* whereas there are generally only two in *Jubula.*

Schuster (1992) recognized only one family, Jubulaceae, with two genera, *Jubula* and *Frullania.* For him, *Schusterella, Steerea,* and *Amphijubula* belong to *Frullania.* Crandall-Stotler and Stotler (2000) included only the genera *Jubula* and *Frullania* in Jubulaceae, merging *Amphijubula, Neohattoria, Schusterella,* and *Steerea* with *Frullania.*

Weis (2001) presented the first cladistic analysis of the suborder Jubulineae based on diverse sporophytic characters. She chose to maintain the assignment of both *Frullania* and *Jubula* to Jubulaceae, although she admitted that their relationships were not clear, based on her cladogram. The Jubulaceae form a paraphyletic group in her majority rule consensus tree.

Frullania is a very large genus with ca. 1000 described taxa (Yuzawa, 1991). Many species are epiphytes, and epiphylls are rare (Schuster, 1980a). Fourteen subgenera have been recognized by different authors (Yuzawa, 1991). *Jubula,* on the other hand, is a rather small genus with only four species (Guerke, 1978). These species are morphologically variable, and this high degree of variability might suggest that species are still actively diverging (Guerke, 1978). Plants usually grow on damp rocks; occasionally they are corticolous or epiphyllous (Schuster, 1980a).

Many authors have thought that *Jubula* is intermediate between *Frullania* and Lejeuneaceae, as *Jubula* and Lejeuneaceae share many morphological characteristics, e.g., the structure of the seta (16 outer and 4 inner cells) and the elaters that are attached to the capsule wall (Spruce, 1884; Schus-

ter, 1980a). Mizutani (1961) even placed *Jubula* and *Neohattoria* in the subfamily Jubuloideae of the Lejeuneaceae together with the genus *Nipponolejeunea* S. Hatt.

LEJEUNEACEAE

This family is very speciose and includes approximately 1400 species distributed across 96 genera (T. Pócs, pers. comm.).

In addition to the main subfamilies Ptychanthoideae, Lejeuneoideae, and Cololejeuneoideae, many small, often monospecific subfamilies have been described. These include the Metzgeriopsioideae, Myriocoleoideae, Tuyamaelloideae, and Nipponolejeuneoideae by Schuster (1963b) and Bryopteridoideae (Stotler) Gradst. by Gradstein (1975). These were later merged with the main subfamilies Lejeuneoideae and Ptychanthoideae (Mizutani, 1985; Gradstein, 1994).

Gradstein (1994, 2001) divided the subfamily Lejeuneoideae into the tribes Lejeuneae, Cololejeuneae (former subfamily Cololejeuneoideae Herzog), and Brachiolejeuneae, and the subfamily Ptychanthoideae into the tribes Nipponolejeuneae and Ptychantheae. Brachiolejeuneae is an interesting transitional taxon with gametophytes reminiscent of those of the Ptychanthoideae and sporophytic characters resembling those of the Lejeuneoideae.

RADULACEAE

The Radulaceae contain 300 species that are all accommodated within a single genus, *Radula* Dumort. (Schuster, 1980a). Plants grow on rock, bark, or leaves. The genus is rather homogeneous, but Castle (1936), following Spruce (1884), divided it into the two subgenera *Cladoradula* and *Acroradula*; Yamada (1979) into the three subgenera *Odontoradula, Radula,* and *Cladoradula*; and Schuster (1980a, 1980b) into four subgenera, *Odontoradula, Radula, Cladoradula,* and *Metaradula.*

The most notable features of the Radulaceae are the rhizoids that are restricted to the leaf lobules, the lack of underleaves, and numerous, pluricellular gemmae on the leaf margins. It is also distinguished from other families by dorsiventrally flattened perianths, *Radula*-type branches and subfloral innovations, by a large, mostly single, olive brown oil body in cells (although many tropical species have one larger and one to several smaller oil bodies) (Yamada, 1979).

The Radulaceae have been considered an isolated family and placed in an order or suborder of their own (Schuster, 1958, 1980a, b; Crandall-Stotler & Stotler, 2000). However, *Radula*-type branches are also found in the Porellales. Chemically, the Radulaceae are one of the most isolated families in the Jungermanniales (Asakawa, 1982).

PTILIDIACEAE

Schuster proposed that *Ptilidium* is a possible ancestor of the Porellinae (Schuster, 1966, 1972, 1980a, 1996), although, according to Schuster (1980a), it is an isolated genus without close relatives. Clearly, the affinities of *Ptilidium,* which accommodates three species, are ambiguous. The family Ptilidiaceae has been defined in different ways (see Müller, 1905–1911; Frye & Clark, 1943; Schuster, 1958, 1966; Grolle, 1983; Crandall-Stotler & Stotler, 2000). *Ptilidium* species often occur as epiphytes on the bark of living trees or on decaying logs.

Materials and Methods

TAXON SAMPLING

The samples represent the Porellaceae (3 species), Frullaniaceae (5 spp.), Jubulaceae (2 spp.), Lejeuneaceae (25 spp.), and Radulaceae (2 spp.) that are or have been included in the order Porellales. Ten new species of Lejeuneaceae are added to the matrix analyzed by Ahonen et al. (2003). *Marchantia polymorpha* L. (Marchantiaceae, Marchantiales), *Ptilidium pulcherrimum* (Weber) Hampe (Ptilidiaceae, Lepicoleales), and *Jungermannia leiantha* Grolle (Jungermanniaceae, Jungermanniales) were used as the outgroups. Of these, *Ptilidium pulcherrimum* has been placed close to the Porellales (e.g., Schuster, 1972, 1980a) or, alternatively, considered to be quite distant from it (Crandall-Stotler & Stotler, 2000). *Jungermannia leiantha* is from the Jungermanniidae, the same subclass as the Porellales, but has not been suggested to be a close relative. *Marchantia polymorpha* is very distantly related, belonging to a different liverwort class (Marchantiopsida). According to Nixon and Carpenter (1993), the role of the outgroup is to establish character polarity, and as long all the ingroup characters are found in outgroup species, the taxonomic distance of the outgroup has no relevance. The voucher specimens for all of these are listed in the Table 1.

The shoots were sampled from material under a dissecting microscope. When possible, only the youngest parts of the shoots were taken, but for very small plants, the whole shoots were used. As many species are extremely small, it was often necessary to use several shoots or individuals. These were always sampled from the same specimen. As the

Table 1. The GenBank accession numbers for the sequences used in the phylogenetic analysis and their respective voucher specimens. Accession numbers for *Marchantia polymorpha* sequences taken from GenBank are shown in italics. Accession numbers marked with asterisks were published in Ahonen et al. (2003).

Taxon	Voucher specimen	GenBank accession no.		
		5.8S-ITS2-26S	*rbc*L	*trn*L-*trn*F
Ingroup				
Frullaniaceae				
Frullania davurica Hampe	Taiwan, *Ahonen, Huttunen & Virtanen 3023*	AY257497	AY302451	AY251177
Frullania dilatata (L.) Dumort.	Finland, *Ahonen & Engblom 15*	AY125333*	AY125929*	AY144469*
Frullania monocera (Hooker & Taylor) Taylor	Taiwan, *Ahonen, Huttunen & Virtanen 3035*	AY257498	AY302450	
Frullania muscicola Steph.	China, *Koponen et al. 56547* (H)		AY302452	AY251178
Frullania tamarisci (L.) Dumort.	Taiwan, *Ahonen, Huttunen & Virtanen 3187*	AY257499	AY302453	AY251179
Jubulaceae				
Jubula hutchinsiae (Hook.) Dumort.	Taiwan, *Ahonen, Huttunen & Virtanen 3190*	AY125350*	AY125946*	AY144477*
Jubula japonica Steph.	China, *Koponen et al. 54308* (H)	AY125342*	AY125938*	AY144479*
Nipponolejeunea pilifera (Steph.) S. Hatt.	Japan, 1999, *Ohnishi s.n.*	AY125341*	AY125937*	AY144478*
Lejeuneaceae				
Aphanolejeunea gracilis Ast	Costa Rica, 22–24 Sep. 2000, *Piippo s.n.*	AY257491	AY302443	AY251169
Austrolejeunea nudipes (Hook. f. & Taylor) Grolle	Australia, *Pócs & Streimann 99147/H* (EGR)	AY257492	AY302445	AY251171
Cheilolejeunea imbricata (Nees) S. Hatt.	China, *Koponen et al. 51583* (H)	AY125339*	AY125935*	AY144475*
Cheilolejeunea inflexa (Hamp ex Lehm. & Lindenb.) Grolle	Ecuador *Norris 91226 & J. Flanagan* (H)	AY257493	AY302446	AY251175
Cheilolejeunea trifaria (Reinw., Blume & Nees) Mizut.	Australia, *Pócs & Streimann 99102/A* (EGR)	AY257494	AY302447	AY251173
Cheilolejeunea trifaria (Reinw., Blume & Nees) Mizut.	Australia, *Pocs 9611/DH* (EGR)	AY257495	AY302448	AY251174
Cololejeunea macounii (Spruce ex Underw.) A. Evans	China, *Koponen et al. 56319* (H)	AY125346*	AY125942*	AY144483*
Cololejeunea peraffinis (Schiffn.) Schiffn.	China, *Koponen et al. 57141* (H)	AY125345*	AY125941*	AY144482*
Drepanolejeunea erecta (Steph.) Mizut.	China, *Koponen et al. 50605* (H)	AY125344*	AY125940*	AY144481*
Drepanolejeunea vesiculosa (Mitt.) Steph.	Australia, *Pócs & Streimann 99122/A* (EGR)	AY257496	AY302449	AY251176
Lejeunea catanduana (Steph.) H. A. Mill., H. Whittier & B. Whittier	China, *Koponen et al. 56008* (H)	AY125348*	AY125943*	AY144484*
Lejeunea cavifolia (Ehrh.) Lindb.	Finland, 1998 *Ahonen s.n.* (H)	AY125347*	AY125945*	AY144486*
cf. *Lejeunea stevensiana* (Steph.) Mizut.	Ecuador, *Norris 90573 & Eduardo Barahona M* (H)	AY257509	AY302463	
Lepidolejeunea bidentula (Steph.) R. M. Schust.	China, *Koponen et al. 51525* (H)	AY125340*	AY125936*	AY144476*
Leptolejeunea elliptica (Lehm. & Lindenb.) Schiffn.	China, *Koponen et al. 50179* (H)	AY125343*	AY125939*	AY144480*
Lopholejeunea brunnea Horik.	China, *Koponen et al. 51570* (H)	AY125334*	AY125930*	AY144470*
Lopholejeunea muelleriana (Gottsche) Schiffn. var. *australis* (Steph.) B. Thiers & Gradst.	Australia, *Pócs & Streimann 99200/T* (EGR)	AY257500	AY302454	AY251180

Table 1. Continued.

Taxon	Voucher specimen	GenBank accession no.		
		5.8S-ITS2-26S	*rbc*L	*trn*L-*trn*F
Mastigolejeunea auriculata (Wilson & Hook.) Schiffn.	China, *Koponen et al. 51516* (H)	AY125337*	AY125933*	AY144473*
Microlejeunea ulicina (Taylor) Steph.	China, *Koponen et al. 49553* (H)	AY125349*	AY125944*	AY144485*
Nephelolejeunea hamata Grolle	Australia, *Pócs & Streimann 99188/B* (EGR)	AY257501	AY302455	AY251181
Odontolejeunea lunulata (F. Weber) Schiffn.	Costa Rica, 22–24 Sep. 2000, *Piippo s.n.* (H)	AY257502	AY302456	AY251182
Ptychanthus striatus (Lehm. & Lindenb.) Nees	China, *Koponen et al. 57218* (H)	AY125335*	AY125931*	AY144471*
Spruceanthus semirepandus (Nees) Verd.	China, *Koponen et al. 50971* (H)	AY125336*	AY125932*	AY144472*
Trocholejeunea sandvicensis MIzut.	China, *Koponen et al. 55768* (H)	AY125338*	AY125934*	AY144474*
Xylolejeunea grolleana (Pócs) X.-L. He & Grolle	Madagascar, *Pócs et Szabó 9878/CY* (H)	AY257509	AY302464	AY251190
Porellaceae				
Porella cordeana (Huebener) Moore	Finland, *Ahonen & Engblom 27*	AY257503	AY302457	AY251185
Porella japonica (Sande Lac.) Mitt.	China, *Piippo 60751* (H)	AY257504	AY302459	AY251183
Porella platyphylla (L.) Pfeiff.	Finland, *Ahonen & Engblom 28*	AY257505	AY302458	AY251184
Radulaceae				
Radula complanata (L.) Dumort.	Finland, *Ahonen & Engblom 14*	AY257507	AY302461	AY251188
Radula okamurana Steph.	Japan, *Ahonen 221*	AY257508	AY302462	AY251189
Outgroup				
Junermanniaceae				
Jungermannia leiantha Grolle	Finland, *He-Nygrén & Piippo 1466*	AY316351	AY149838	AY149857
Marchantiaceae				
Marchantia polymorpha L.	Sone et al. (1999), Yamano et al. (1984)	*AB021684*	*Y00686*	*X04465*
Ptilidiaceae				
Ptilidium pulcherrimum (Weber) Hampe	Finland, *Ahonen & Engblom 7*	AY257506	AY302460	AY251186

Porellales often are epiphytes and epiphylls, care was taken to avoid contamination from the attached substratum.

DNA extraction, PCR, and sequencing were done as in Ahonen et al. (2003). The following primers were used: *trn*L-*trn*F: *trn*C and *trn*F (Taberlet et al., 1992), ITS2: 5.8SR (⟨http://www.biology.duke.edu/fungi/mycolab/primers.htm⟩), and LS4R (Shaw, 2000), *rbc*L: *rbc*LF and *rbc*LR (Ahonen et al., 2003).

CLADISTIC ANALYSES

The simultaneous parsimony analysis was performed using direct optimization (Wheeler, 1996) as implemented in the program POY version 3.0. (Wheeler et al., 2002). In this procedure the search for the shortest tree and alignment of sequences are done simultaneously. Insertions and deletions are treated as processes and sequence homologies are dynamically determined and uniquely tailored for each phylogeny (Wheeler, 2001). The length of the cladogram is the sum of all the necessary substitution and insertion/deletion events to produce the tree in question, using the gap, transition, and transversion costs set by the user. In an average analysis, several million different character optimizations are evaluated in order to find the most parsimonious one. For more detailed explanation of the procedure see Wheeler (1996) and Schulmeister et al. (2002).

Cutting sequences into shorter fragments can save dramatically on computation time and space when using optimization alignment (Wheeler et al., 2002). In order to cut the sequences to such subfragments the sequences were first aligned with ClustalW version 1.8 (Thompson et al., 1994). The length variation at the beginning and end of the sequences was filled with Xs, to avoid leading and trailing gaps, which would require a special cost set to them (Schulmeister et al., 2002), and having to assess the effects of changes in this parameter value as well. POY reads the X as an unknown base or a gap. The sequences were cut into subfragments, each defined at both ends by conserved sites, where their homology was certain. The gaps were then removed from the subfragments. Giribet (2001) has noted that cutting the data into subfragments, and the number of such subfragments, can affect the resulting tree lengths. In addition, it introduces an extra degree of subjectivity to the analysis. However, the reduction of the computer time thus achieved is necessary to perform extensive searches with the existing hardware. The analyses took 18–24 hr. each on a one-processor (2.2 GHz) PC. All the analyses were unconstrained. The POY command line was as follows:

```
poy -molecularmatrix Filename
-norandomizeoutgroup -holdmaxtrees 20 -maxtrees
5 -fitchtrees -multibuild 10 -random 5 -seed -1
-slop 3 -checkslop 30 -tbr -spr -sprmaxtrees 5
-drifttbr -numdrifttbr 5 -ratchetspr 5 -fuselimit 50
-fusingrounds 1 -impliedalignment -indices
-repintermediate -poytreefile Filename
-poybintreefile Filename -phastwincladfile
Filename Inputfiles >Outfile.out
```

Information about these commands can be found in the POY manual, available from the American Museum of Natural History website: ⟨//ftp.amnh.org/pub/people/wheeler/poy/poy.pdf⟩.

The cost parameters chosen can have a great effect on results (e.g., Giribet, 2001; Schulmeister et al., 2002). Because there are no empirical data that would unequivocally suggest a suitable parameter combination to certain data a priori, Wheeler (1995) proposed sensitivity analysis as one solution to the problem. In sensitivity analysis the alignments and trees produced with different gap costs are evaluated by their congruence either with accepted monophyletic groups based on morphology (taxonomic congruence sensu Wheeler, 1995, not Miyamoto & Fitch, 1995) or by the congruence of different data subsets used in the same analysis (Wheeler 1995, 2001). The latter is analogous for looking for greatest congruence among characters. The congruence between data sets is assessed by the ILD (incongruence length difference) test (Mickevich & Farris, 1981; Farris et al., 1995) using the formula:

$$\text{ILD} = \frac{(\text{length of the combined tree} - \Sigma\ \text{length of individual trees})}{(\text{length of the combined tree})}$$

In the present study, six different parameter combinations of gap : transversion : transition costs were examined:

(1) Equal costs 1 : 1 : 1. According to some authors, this is the most parsimonious assumption, as no unproven assumption of the frequency of different transition or indel events is implemented (e.g., Frost et al., 2001).

(2) 2 : 2 : 1. This was invoked to explore how equalizing the costs of transversions and of indel events rather than the more usual manner of equalizing transversions and transitions would affect the results.

(3) 3 : 2 : 1. These parameter values reflect the idea that transitions would be more frequent than

transversions (Fitch, 1967; Brown et al., 1982; Li et al., 1984) and transformations in general more frequent than deletions and insertions. The idea that transversions would be more common than transitions is included, for example, in models of base substitutions widely used in evolutionary studies (e.g., Kimura, 1980, 1981; Blaisdell, 1985). Setting gap cost higher than transformation cost is usual in phylogenetic studies, and this is the default in POY.

(4) 2:1:1, 4:1:1, and 8:1:1. Earlier results suggest that gap cost variation affects the topology more than transformation costs when combined molecular data sets are analyzed (Wheeler & Hayashi, 1998). To map this effect, three different gap costs were tested, while the transformation costs were kept equal, setting the parameter values to 2:1:1, 4:1:1, and 8:1:1.

The six parameter combinations described above were of course only a small subset of the whole parameter space, but they covered most values typically used.

To examine the effect of different parts of the data on the resulting topologies, different fractions of data were removed and the remainder was studied with the parameter values that gave the best congruence for the whole data, 1:1:1 and 2:2:1.

(5) ITS2 removed. First, the ITS2 region was removed, leaving, however, the partial 26S and 5.8S genes surrounding it. ITS2 is a region that has been assumed to evolve very fast (Baum et al., 1998), and in many studies it has been found to be best suited for studies at genus or species level (e.g., Mindell & Honeycutt, 1989; Zambino & Szabo, 1993; Kim & Jansen, 1994). Fast-evolving, saturated gene regions or the third positions in coding genes have been regarded as sources of homoplastic noise (e.g., Hillis, 1991) that should be removed from the analysis to bring forth the true signal (Mindell & Honeycutt, 1990; Swofford et al., 1996; Naylor & Brown, 1997), particularly if these regions cannot be aligned unambiguously. However, recent evidence suggests that the amount of homoplasy normally present in data sets does not conceal the true signal (Wenzel & Siddall, 1999) and that homoplasy actually increases the phylogenetic structure (Källersjö et al., 1999; Broughton et al., 2000). Here, the effect of the ITS2 region on the level of homoplasy, character congruence, and the phylogenetic structure was tested.

(6) Region corresponding to the shared deletion in *trn*L intron of *Marchantia polymorpha* and *Cheilolejeunea trifaria* (100 bp) removed. With all gap costs except one, the outgroup *Marchantia polymorpha* detached from the root and was drawn to *Cheilolejeunea trifaria* clade, indicating a putative case of long branch attraction. Both Caterino and Vogler (2002) and Belshaw and Quicke (2002) have reported cases when higher gap costs used in the optimization alignment caused unrelated sequences with independent indels to attract each other in a cladogram. The *trn*L intron of both *M. polymorpha* and *C. trifaria* are much shorter (234 base pairs [bp] and 232 bp, respectively) than the intron of any other species in this study (from 268 bp in *Jungermannia leiantha* to 368 bp in *Frullania davurica*), including the other *Cheilolejeunea* Spruce (Schiffn.) species (335 and 336 bp). A shared deletion of about 100 bp appears in both *M. polymorpha* and *C. trifaria* in the second half of the intron, between clearly distinguished conservative regions. The exact length of the deletion depends of the alignment in this particular region.

To explore the effect of this shared deletion on the placement of *M. polymorpha*, the corresponding gene region was removed from all the sequences. Because in the foregoing analyses it had become quite obvious that the peculiar placement of *M. polymorpha* was not caused by ITS2 (removal of ITS2 had no effect on this placement, see section Results below), the test was performed with the smaller data set without ITS2 in order to save computation time.

(7) Fixed states method. According to Wheeler (1999), the fixed states method should be less susceptible to branch attraction, because the diversity of character states (every sequence is a character state) in this method would greatly reduce the possibility of random character state matching. To test this, the data set without ITS2 was analyzed with the parameter values 1:1:1 using the fixed states method (Wheeler, 1999). The fixed states analysis was run with the same command line as the optimization alignment, except that command **–fixed-states** was added.

(8) Separate analyses of individual genes. Each of the three genes was analyzed also separately, using the parameter values given above, to get the necessary tree lengths for ILD tests (see above).

In order to assess stability of the trees, the Bremer support values (Bremer, 1988) were calculated using the binary representation of the tree with the command line:

```
poy -molecularmatrix matrixfile inputfiles -seed -1
-replicates 1 -topology "(binary representation of
the tree);" -norandomizeoutgroup -bremer >
outfile.
```

The Bremer support values obtained this way might be overestimates, because these values are based on a single tree bisection-reconnection (TBR)

branch swap sample. However, these Bremer support values are sufficiently accurate to estimate the relative support for branches in individual trees, and more importantly to distinguish those that lack it (Grant & Kluge, 2003).

Results

MOLECULAR MARKERS

Three genetic loci were sampled for all species included in this study. The GenBank accession numbers for the sequences are given in Table 1. A BLAST search was performed for each sequence to make sure it was of bryophyte origin. The percentage of missing data in the whole data set was about 2.5%. The gaps resulting from length variation due to insertions and deletions were treated as fifth character states in the analyses.

ITS2. This nuclear gene region consists of the internal transcribed spacer II and the partial sequences of 26S and 5.8S ribosomal RNA genes surrounding it. The length of the complete sequences varied from 688 (*Porella platyphylla* (L.) Pfeiff.) to 884 base pairs (*Spruceanthus semirepandus* (Nees) Verd.). There were some difficulties in sequencing *Cololejeunea peraffinis* (Schiffn.) Schiffn. and *Aphanolejeunea gracilis* Ast, and consequently their ITS2 sequences were only 409 bp and 362 bp long, respectively.

rbc*L.* A partial sequence of the large subunit of the ribulose bisphosphate carboxylase (*rbc*L) gene from the chloroplast was used. The fragment was 1042 base pairs long with no natural length variation. Due to sequencing difficulties, the sequence of *Cheilolejeunea trifaria* was only 500 bp, that of *Drepanolejeunea vesiculosa* 1004 bp, and that of *Frullania monocera* 981 bp long.

trn*L.* This chloroplast fragment contains a partial sequence of the leucine transfer RNA (UAA), an intergenic spacer, and a partial sequence of phenylalanine transfer RNA (GAA). Leucine transfer RNA contains two exons and an intron between them. The length of the complete sequences varied from 396 (*Cheilolejeunea trifaria*) to 587 bp (*Frullania davurica* Hampe) due to differences in the length of the variable regions and the intergenic spacer.

SIMULTANEOUS ANALYSIS

Tree obtained with the parameter costs 1:1:1. In the simultaneous analysis of all the data, the best tree in terms of both congruence between data subsets (ILD value 0.033) and congruence of the tree topology with accepted monophyletic groups from earlier studies was the tree obtained with the parameter values (gap:transition:transversion costs) 1:1:1 (Fig. 1). Also, the consistency index (CI) and retention index (RI) were the highest in this tree (0.52 and 0.72, respectively, Table 2). This is congruent with the result obtained earlier with smaller data set of 18 species and three genomic regions (Ahonen et al., 2003). The 1:1:1 tree is second best regarding the percentage of informative characters (40.9%) of the alignment (Table 2). The differences in the percentages of informative characters are, however, surprisingly small and perhaps insignificant between different alignments, although the length of sequence alignments varies by several hundred base pairs (Table 2). Contrary to what Caterino and Vogler (2002) found in their study, increasing the gap costs did not linearly increase the number of informative characters (Table 2).

In the tree obtained with equal costs (Fig. 1), the ingroup was divided in two sister clades. The first comprises *Ptilidium pulcherrimum,* as a sister taxon to a clade composed of *Porella, Radula,* and *Frullania.* In the *Frullania* clade, *F. tamarisci* from the subgenus *Frullania* Raddi is sister to the species from the subgenus *Trachycolea* Spruce.

The other clade contains *Nipponolejeunea pilifera,* as sister to *Jubula,* and the Lejeuneaceae. In the Lejeuneaceae, *Odontolejeunea lunulata* is most basal, and the remaining taxa are divided two sister clades, corresponding to the Ptychanthoideae and the Lejeuneoideae. In the Ptychanthoideae, *Trocholejeunea sandvicensis* is most basal. *Spruceanthus semirepandus* and *Ptychanthus striatus* group together in apical position with *Lopholejeunea brunnea* as their sister. *Lopholejeunea* (Spruce) Schiffn. is resolved as polyphyletic. In the Lejeuneoideae clade, *Drepanolejeunea* (Spruce) Schiffn. from the tribe Lejeuneae is monophyletic and sister to the remainder of the subfamily. The latter are divided in two sister clades, one containing the Tuyamaelloideae and Cololejeuneae species and the other comprising species of the Lejeuneae. In the Tuyamaelloideae–Cololejeuneae clade, *Nephelolejeunea hamata* and *Austrolejeunea nudipes* from Tuyamalloideae form a grade leading to a monophyletic clade composed of *Cololejeunea macounii, C. peraffinis,* and *Aphanolejeunea grolleana* from Cololejeuneae.

In the clade with the remaining Lejeuneae, two main clades are resolved: one contains *Lejeunea* (three species) and *Microlejeunea ulicina,* and the other *Xylolejeunea grolleana, Leptolejeunea elliptica,* and a monophyletic *Cheilolejeunea* clade (three

Table 2. Attributes of trees obtained with different cost parameters and with different portions of the data in parsimony analysis. In fixed states method whole sequences are treated as character states, and thus CI and RI values or number of informative characters cannot be calculated for trees obtained with this method. CI = consistency index, RI = retention index, ILD = incongruence length difference.

gap:transversion:transition cost ratios	Length of trees	Trees obtained	CI	RI	ILD	No. of informative sites/ total no. of characters (bp)	Percentage of informative characters
1:1:1	5858	1	0.52	0.72	0.033	1455/3559	40.9%
2:2:1	9625	1	0.50	0.72	0.047	1432/3487	41.1%
3:2:1	11808	1	0.46	0.71	0.057	1302/3300	39.5%
2:1:1	8078	1	0.45	0.70	0.053	1324/3210	41.24%
4:1:1	11723	1	0.40	0.68	0.096	1226/3526	40.51%
8:1:1	17775	1	0.34	0.63	0.15	1156/2918	39.6%
1:1:1 excluding ITS2	2550	3	0.49	0.73	0.04	700/2273	30.8%
1:1:1 excluding ITS2 and *trn*L indel (100 bp)	1745	6	0.51	0.75	0.033	517/1987	26.0%
1:1:1 excluding ITS2 Fixedstates	2793	20			0.019		
2:2:1 excluding ITS2	3927	1	0.48	0.73	0.04	707/2266	31.2%
2:2:1 excluding *trn*L indel	8156	1	0.50	0.72	0.039	1187/3173	37.4%

species). *Lepidolejeunea bidentula* is sister to these two main clades combined.

2:2:1 tree. Next best tree according to ILD values (0.047) is the one obtained with gap:transition:transversion costs 2:2:1 (tree not shown). CI is 0.50 and RI = 0.72 (Table 2).

In this tree, like in all trees obtained with gap cost higher than one, *Marchantia polymorpha* is removed from the root and drawn to the *Cheilolejeunea trifaria*. If the tree is still rooted with *M. polymorpha* (rather than with *Jungermannia leiantha*), the whole phylogeny of the Porellales is turned upside down compared to 1:1:1 tree, and also compared to all the earlier concepts of Porellales evolution (e.g., Schuster, 1980; Crandall-Stotler & Stotler, 2000; Gradstein et al., 2003). Therefore, for the sake of clarity, the tree is described here rooted with the other outgroup, *J. leiantha*. This topology is more congruent with the results of earlier studies based on morphology (e.g., Schuster, 1980; Crandall-Stotler & Stotler, 2000; Gradstein et al., 2003), especially in the basal regions of the tree, than the tree obtained with parameters 1:1:1.

Ptilidium pulcherrimum is resolved as an outgroup, occupying a position that is sister to Porellales–Radulales. The monophyletic *Porella* is sister to a clade wherein *Radula* is sister to the remaining species, which are divided in two sister clades. The first includes a monophyletic *Frullania*. The second clade is divided into a *Jubula–Nipponolejeunea* clade and a Lejeuneaceae clade. *Odontolejeunea lunulata* is again sister to a monophyletic lineage composed of two clades, corresponding to the Ptychanthoideae and the Lejeuneoideae. The relationships in the Ptychanthoideae clade are as in the 1:1:1 tree. The Lejeuneoideae are resolved in two clades, one with the Tuyamaelloideae and Cololejeuneae, the other with Lejeuneae. The relationships within Tuyamaelloideae–Cololejeuneoideae clade are the same as in the tree obtained with equal weights. In the Lejeuneae clade, *Lepidolejeunea bidentula* is most basal, and the rest is divided to two clades. One of them contains *Drepanolejeunea, Lejeunea* Libert., and *Microlejeunea ulicina*, and the other *Xylolejeunea grolleana, Leptolejeunea elliptica, Cheilolejeunea,* and *Marchantia polymorpha* as a sister to *C. trifaria* clade.

2:1:1 tree. The ILD value of this tree is 0.053, CI is 0.45, and RI = 0.70 (tree not shown, Table 2). *Marchantia polymorpha* is drawn to *Cheilolejeunea trifaria*, so the tree is described here rooted with *Jungermannia* L.; *Ptilidium pulcherrimum* is again in an outgroup position. *Porella* is the basalmost ingroup taxon, and *Frullania* and *Radula*

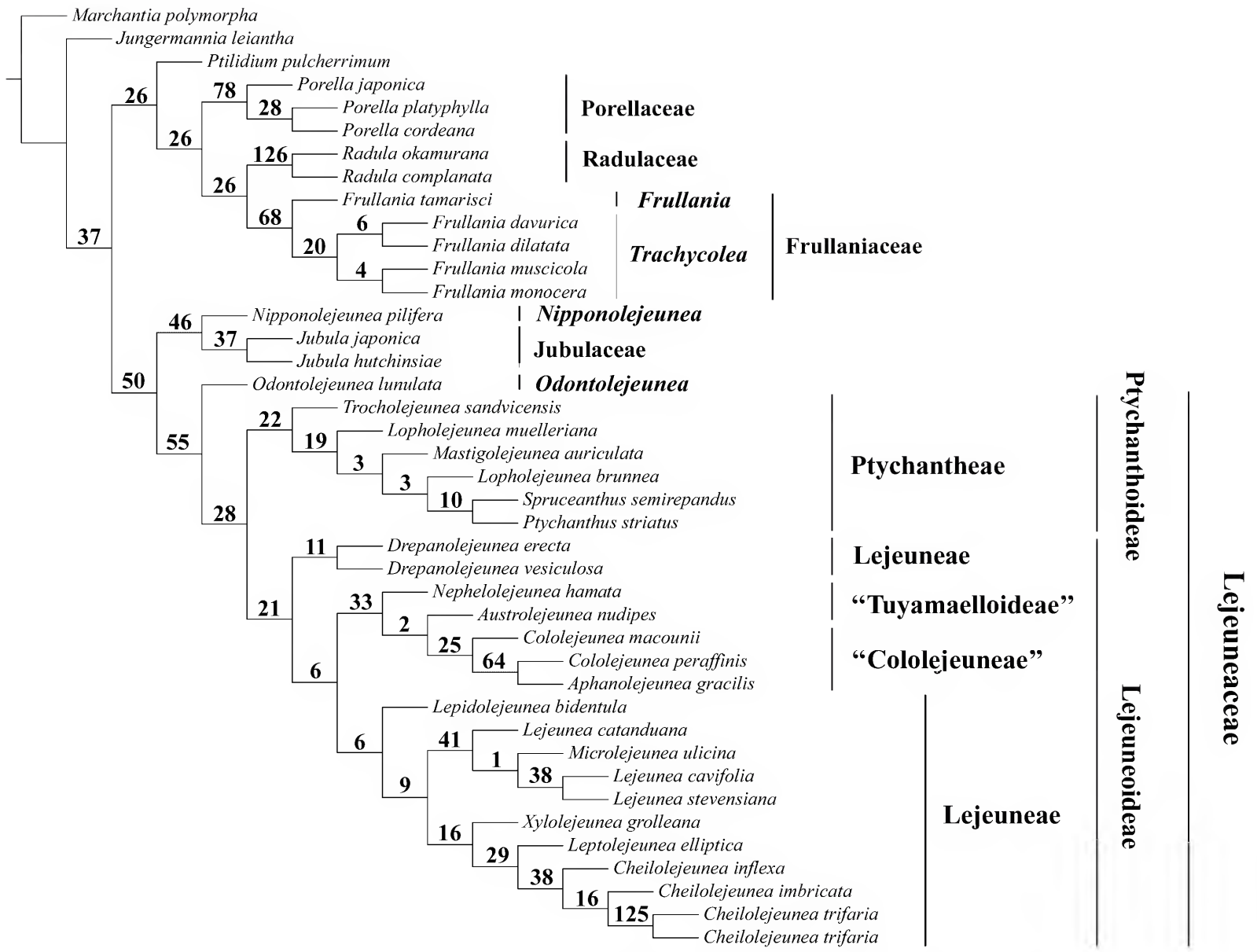

Figure 1. The most congruent tree judged by the ILD values was obtained with the parameter values (gap : transition : transversion costs) 1 : 1 : 1. Taxonomic groupings based on earlier morphological studies are shown beside the tree. Subfamily Tuyamaelloideae R. M. Schust. and tribe Cololejeuneae Gradstein appear in quotes, because, although the clades are monophyletic, recognizing them in this tree would leave the Lejeuneoideae paraphyletic. The numbers above the nodes are the Bremer support values. For tree length and other attributes, see Table 2.

form a grade leading to *Jubula–Nipponolejeunea* clade. The *Jubula–Nipponolejeunea* clade is sister to the Lejeuneaceae. *Odontolejeunea lunulata* is sister to the rest of the Lejeuneaceae. These are divided in a Tuyamaelloideae–Cololejeuneae clade and a Lejeuneoideae–Ptychanthoideae clade. In this clade *Lejeunea* and *Microlejeunea ulicina* compose a basal monophyletic lineage. *Drepanolejeunea erecta* and *Xylolejeunea grolleana* form a grade leading to the main two clades. One of them contains *Lepidolejeunea bidentula* (Lejeuneae) as the basal most taxon, and *Trocholejeunea sandvicensis, Lopholejeunea brunnea, L. muelleriana, Mastigolejeunea auriculata, Spruceanthus semirepandus, and Ptychanthus striatus* (Ptychanthoideae). In the last clade *Leptolejeunea elliptica* is sister to the *Cheilolejeunea* species and *Marchantia polymorpha.*

3 : 2 : 1 tree. The ILD value of this tree is 0.057, CI is 0.46, and RI = 0.71 (tree not shown, Table 2). *Marchantia polymorpha* is drawn to *Cheilolejeunea trifaria,* so the tree is described here rooted with *Jungermannia* L. *Ptilidium pulcherrimum* is in outgroup position, *Porella* and *Radula* form a grade leading to a clade containing Frullania as sister to a clade containing monophyletic *Jubula–Nipponolejeunea* and Lejeuneaceae clades. *Odontolejeunea lunulata* is sister to the rest of the Lejeuneaceae. *Cololejeunea peraffinis–Aphanolejeunea gracilis* clade, *Cololejeunea macounii* (Cololejeuneae), *Austrolejeunea nudipes–Nephelolejeunea hamata* (Tuyamaelloideae) clade, *Drepanolejeunea erecta,* monophyletic *Lejeunea–Microlejeunea* clade, and *Xylolejeunea grolleana* (Lejeuneae) form a grade leading to the two main clades. One of them contains Ptychanthoideae with basal *Lepidolejeunea bidentula* and the other *Leptolejeunea elliptica* basal in *Cheilolejeunea–Drepanolejeunea vesiculosa–Marchantia polymorpha* clade.

4 : 1 : 1. tree. The ILD value of this tree is 0.096, the CI is 0.40, and the RI = 0.68 (tree not shown, Table 2). *Marchantia polymorpha* is again sister to *Cheilolejeunea trifaria,* so the tree is de-

scribed here rooted with *Jungermannia*. The tree is composed of two sister clades. One comprises *Radula* and *Porella*. The other includes *Ptilidium pulcherrimum*, which is resolved as a sister taxon to the *Frullania–Jubula*–Lejeuneaceae clade. *Nipponolejeunea* and *Jubula* form a monophyletic lineage that is sister to the Lejeuneaceae. Within the latter the Cololejeuneae and the Lejeuneae are polyphyletic, and so are *Cololejeunea* and *Lejeunea*. *Cololejeunea peraffinis* and *Aphanolejeunea grolleana* (Cololejeuneae) form a basal lineage that is followed by a grade composed of *Odontolejeunea lunulata* (Brachiolejeuneae), *Austrolejeunea nudipes*, *Nephelolejeunea hamata* (Tuyamaelloideae), *Cololejeunea macounii* (Cololejeuneae), the *Microlejeunea ulicina–Lejeunea catanduana* clade, *Drepanolejeunea erecta*, and *Xylolejeunea grolleana* (Lejeuneae). The remaining taxa are distributed in two sister clades. The first one includes *Cheilolejeunea*, *Marchantia polymorpha*, *Leptolejeunea elliptica*, and *Drepanolejeunea vesiculosa* (Lejeuneae). The sister clade contains *Lejeunea cavifolia* and *L. stevensiana* (Lejeuneae) as sister to *Lepidolejeunea* (Lejeuneae)–Ptychanthoideae clade.

8:1:1. tree. The ILD value of this tree is 0.15, the CI is 0.34, and the RI = 0.63 (tree not shown, Table 2). *Marchantia polymorpha* is sister to *Cheilolejeunea trifaria*, so the tree is described here rooted with *Jungermannia*. *Frullania tamarisci* is the basalmost taxon, followed by a lineage composed of the remaining species of the *Frullania* and then *Radula*. The remaining Porellales are divided in two sister clades. One contains *Cololejeunea peraffinis*, *Aphanolejeunea grolleana*, *Ptilidium pulcherrimum*, and *Porella*. In the other clade *Jubula* is sister to the Lejeuneaceae. *Nipponolejeunea pilifera* (Ptychanthoideae), *Austrolejeunea nudipes* (Cololejeuneaea), *Odontolejeunea lunulata* (Brachiolejeuneae), *Lopholejeunea brunnea*, *Trocholejeunea sandvicensis*, and *Lopholejeunea muelleriana* (Ptychanthoideae) compose a basal grade in Lejeunaecae. The remaining Lejeuneaceae are divided in two clades, one containing *Lepidolejeunea bidentula*, *Xylolejeunea grolleana*, *Drepanolejeunea erecta*, *Microlejeunea ulicina*, *Lejeunea catanduana* (Lejeuneae), *Nephelolejeunea hamata* (Tuyamaelloideae), and *Cololejeunea macounii* (Cololejeuneae). The other clade contains *Mastigolejeunea auriculata*, *Ptychanthus striatus*, *Spruceanthus semirepandus* (Ptychanthoideae), *Leptolejeunea elliptica*, *Lejeunea cavifolia*, *L. stevensiana*, *Drepanolejeunea vesiculosa*, and paraphyletic *Cheilolejeunea*, as well as *Marchantia polymorpha*.

The effect of homoplasy: ITS2 removed. Removal of the ITS2 data sets worsens the ILD value when the cost parameters 1:1:1 were used (0.04 ITS2 excluded), but improves congruence when the parameters 2:2:1 were used (0.04 ITS2 excluded). CI and RI get worse in both cases, and the percentage of informative characters clearly decreases (Table 2). The congruence with the results based on morphology remains good in both cases, and, in the case of cost parameter values 1:1:1, congruence with morphology actually increases in the basal nodes of the tree (Fig. 2). With parameter values set at 1:1:1 three trees were obtained, which differ only in the branching order inside the Ptychanthoideae clade (Fig. 2).

With both parameter settings *Marchantia polymorpha* groups with *Cheilolejeunea* and the *Drepanolejeunea* clade dissolves, indicating that the putative long branch attraction is not due to the homoplasy and saturation in ITS2 region. Indeed, when the ITS2 is removed, *M. polymorpha* groups with *C. trifaria* even with the parameters set at 1:1:1 (Fig. 2a), suggesting that there is something in the ITS2 region that counteracts this topology. Probably the longer alignment of ITS2 that was produced when gap cost was set equal to transformation costs contains enough synapomorphies to outweigh the attraction effects from other parts of the data.

Indel attraction. Removing the region corresponding to the 100 bp deletion in *trn*L intron of *Marchantia polymorpha* and *Cheilolejeunea trifaria* from all the taxa maintains *M. polymorpha* in an outgroup position, whether the parameter values are set at 1:1:1 (Fig. 2b) or 2:2:1 (tree not shown). However, although removing this region had a positive effect on the placement of *M. polymorpha* and on character congruence as measured by ILD values (0.033 for 1:1:1 tree and 0.039 for 2:2:1 tree), the congruence with previous morphological studies (Schuster, 1980; Crandall-Stotler & Stotler, 2000; Gradstein, 2001; Gradstein et al., 2003) decreased. It is possible that the sites corresponding to the deletions contained important phylogenetic signal for sequences from the other taxa, which was lost when this region was removed.

Indel repulsion. Surprisingly, in addition to the branch attraction effects, the *trn*L indel region seems to have a quite opposite, repulsive effect on the two *Drepanolejeunea* species. The pattern is in direct contrast to how *Cheilolejeunea trifaria* and *Marchantia polymorpha* are resolved. The *Drepanolejeunea* clade dissolves with high gap costs, and if the ITS2 region is removed, but returns together if the *trn*L indel is removed. Thus, in this case the indel region seems to contain something that repels, rather than attracts. The *Drepanolejeunea* sequenc-

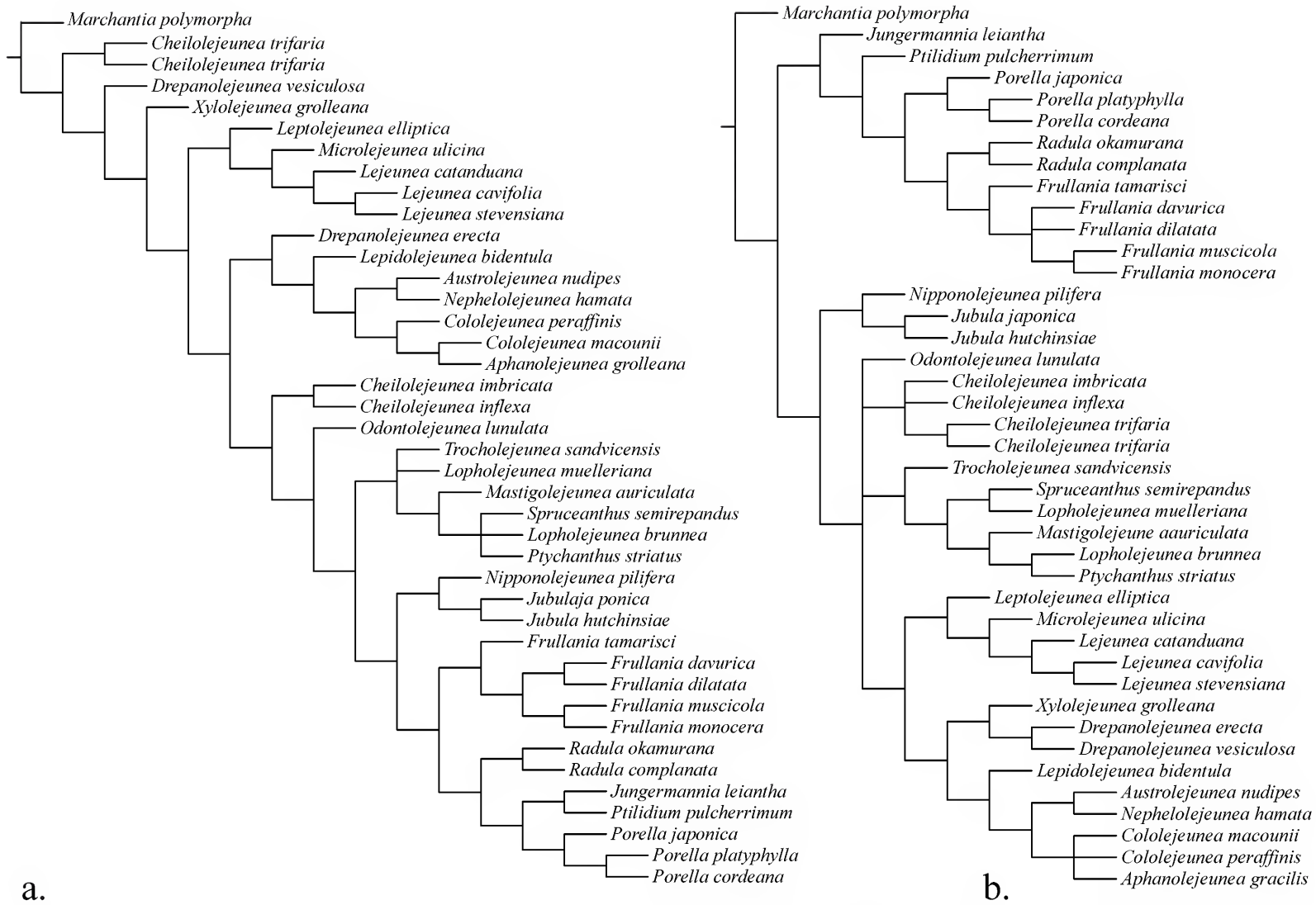

Figure 2. —a. Consensus of the three 1:1:1 trees obtained without ITS2 data. When the highly variable ITS2 data set was removed from the analysis, *Marchantia polymorpha* was drawn to *Cheilolejeunea trifaria* even with parameters 1:1:1. —b. When the region corresponding to the deletions of 100 base pairs in the *trn*L intron in *M. polymorpha* and *C. trifaria* was removed from all the sequences in the analysis, in addition to ITS2 region, *M. polymorpha* was dissociated from *C. trifaria*. Consensus tree of six most parsimonious trees. Parameter values were 1:1:1. Numbers above the nodes are Bremer support values. For tree length and other attributes of the trees, see the Table 2.

es in this part are, indeed, different from each other, *D. vesiculosa* sequence having a deletion of about 14 bp. *Drepanolejeunea vesiculosa* also has an autapomorphic insertion of about 20 bp in the end of the intron. Probably, without the opposing synapomorphies from ITS2 region, these differences prevent *Drepanolejeunea* species from grouping together.

Fixed states method. *Marchantia polymorpha* was drawn to the *Cheilolejeunea* clade even with the fixed states method when ITS2 region was omitted and the data were run with the parameter values 1:1:1. Fixed states method produced 20 trees (trees not shown), in all of which *Frullania* was sister to the rest of the Porellales. Next basal most were the *Ptilidium–Radula–Porella* clade and the *Nipponolejeunea–Jubula* clade. Inside Lejeuneaceae, the Ptychanthoideae and Lejeuneoideae were sister clades. *Odontolejeunea lunulata* was sister to the rest of Lejeuneoideae in all 20 trees. Inside Lejeuneoideae, the structure of trees varied very much, the only constant features being the monophyly of Tuyamaelloideae–Cololejeuneae clade, *Lejeunea–Microlejeunea* clade, and *Cheilolejeunea–Drepanolejeunea vesiculosa–Marchantia polymorpha* clade.

Discussion

Methodology

Many studies have demonstrated the effect of the costs set for substitutions and indel events on the topology of the trees (e.g., Wheeler, 1995; Phillips et al., 2000; Giribet, 2001; Schulmeister et al., 2002; Ahonen et al., 2003). This effect was also observed in the present study (Fig. 3). Sensitivity analysis (Wheeler, 1995), i.e., looking for the maximum congruence either between different parts of data or with previous studies, has been proposed as one way to choose among different trees. However, choosing the favored result based on congruence with the results of earlier studies seems to be in conflict with the basic philosophy of science, which states that theories can be falsified or confirmed by new results, but the new results cannot be confirmed or falsified by prevailing theories.

Even Wheeler himself later argued (Giribet & Wheeler, 1999: 135) "Taxonomic congruence as used by Wheeler (1995) is probably not a good criterion to choose among competing hypotheses because the groups have to be defined arbitrarily (perhaps based on current taxonomy). However, taxonomic congruence can be very informative in comparing some taxonomic groups to hypotheses obtained."

Looking for congruence between different parts of the data seems a better method in this respect. The justification for this is that if we are looking for a cladogram indicating the greatest congruence among characters (i.e., the most parsimonious tree), we can extend this requirement for congruence also to among-data set comparisons (Phillips et al., 2000). Actually, Wheeler calls the congruence among data the fulcrum of parsimonious phylogenetic method (Wheeler, 2001). However, there are studies that claim the contrary, that the best ILD values do not reflect the accuracy of the topology (Yoder et al., 2001; Dowton & Austin, 2002).

Usually the congruence of different genes is compared, but genes as units are in fact arbitrary (Kluge, 1998). The genes often contain very different regions, e.g., exons, introns, loops, etc., and perhaps the parameters should be explored using each of these regions as separate units. Vidal and Lecointre (1998) found that intragenic incongruence is in some cases higher than intergenic incongruence. Nevertheless dividing every gene to smaller partitions would lead to even more computational time, and it would be difficult (practically impossible) to find the smallest region, for which we can assume evolution to have been uniform.

If the criterion of maximal congruence is accepted, in the present study the most congruent results are obtained with the weighting 1:1:1, when the combined data are used, and with weighting 2:2:1, if the ITS2 is removed (Table 2). In Schulmeister et al. (2002), the best values for simultaneous analysis of molecular data and morphology were 4:1:1, but for the molecular data alone the best values were 1:1:1. Cognato and Vogler (2001) found that the parameters 2:1:1 were the best choice, whereas in Frost et al. (2001) 1:1:1:1 offered the most congruent results. It seems, thus, that no values can be recommended universally. This is also what Giribet (2001) concluded when he assessed the best parameter combinations for three data sets under different conditions. Broughton et al. (2000) discovered in their study that transitions provided substantially more phylogenetic information up to about 25% divergence. However, Broughton et al. found little benefit of using 2:1 transversion:transition cost and recommended equal weighting.

In the present study, *Marchantia polymorpha* is unexpectedly detached from the root and groups with *Cheilolejeunea trifaria* if gap costs higher than one are used. It is hardly possible that this could be correct placement for *M. polymorpha,* which belongs to a different liverwort class (Crandall-Stotler & Stotler, 2000). Caterino and Vogler (2002) found that independent insertions in different sequences could cause long branch attraction in direct optimization. In their study, with higher gap costs, the sequences seemed to group together largely according to their length. Belshaw and Quicke (2000) have found a similar effect with unrelated insertions on their results obtained with POY.

The phenomenon described in those studies seems to be the best explanation also for the placement of *Marchantia polymorpha* in the present study. *M. polymorpha* and *C. trifaria* grouped together in the 2:2:1 analysis. The removal of the highly variable ITS2 region from the analysis did not have any affect on the position of *M. polymorpha,* whereas its removal in the 1:1:1 analysis brought *M. polymorpha* and *C. trifaria* together even with those paramters. The attraction was, thus, clearly not due to an essential difference in sequences, but to some effect that the ITS2 region had counteracted in the 1:1:1 total data tree. A deletion of 100 base pairs was found in *trn*L intron in both *M. polymorpha* and *C. trifaria.* It is plausible that the deletions in *M. polymorpha* and *C. trifaria* are independently acquired, as the other parts of the sequences do not bear any close similarity to each other. Indeed, when the corresponding part of the intron was removed from all the species, *C. trifaria* detached from *M. polymorpha.*

The proneness of parsimony methods to long branch attraction (Felsenstein, 1978) has been a controversial issue. Advocates of parsimony have denied the seriousness of this phenomenon (e.g., Siddall, 1998; Siddall & Whiting, 1999; Pol & Siddall, 2001), and perhaps in some cases it truly has been used as a convenient way to dismiss undesirable results. In the present case, the long branch attraction appears to be a consequence of treating gaps as fifth base in POY. When the gaps are treated as fifth base, the shared deletions become synapomorphies of the taxa that have them. Thus, if there are not enough correct synapomorphies to overcome their effect, the branches are drawn together.

Although removing the *trn*L indel had an advantageous effect of removing the attraction and the

trees obtained without it had high ILD values, the topology became less congruent with earlier results (Fig. 2). It is not possible to know which one is the right result, but it is possible that the sites corresponding to the deletions contained important phylogenetic information in the other sequences, which was lost when this region was removed. Thus, simply removing such indel regions is not a good solution. Adding data either in the form of new species or more characters would probably be the safest approach to resolve this problem. According to Graybeal (1998), adding taxa to analysis improves the accuracy of the phylogenetic analysis and attenuates long branch attraction, while Poe and Swofford (1999) concluded that adding taxa can also lead to wrong phylogenies and recommended instead adding characters. Here the latter seems to work, as ITS2 region alone could inhibit the attraction resulting from length differences in *trn*L.

The case of branch repulsion in the *Drepanolejeunea* species seems to be due to the same indel-region in the *trn*L intron as the foregoing branch attraction. In this case, the region seems to contain something that prevents the *Drepanenolejeunea* species from grouping together. The introns of these species were found to be of different length, and the effect is probably due to an insertion and a deletion that has only happened in *D. vesiculosa*. Contrary to long branch attraction, long branch repulsion (sister taxa both with long branches repulse each other) has been described to be a problem of maximum likelihood methods (Siddall, 1998; Pol & Siddall, 2001).

Indel attraction and repulsion could also be responsible for other strange groupings in the trees obtained with high gap costs, especially values of 4 and 8, although no attempt was made to trace the origins of those groupings. Cost parameters set at 1:1:1 seems to avoid the problems described above.

It seems that homoplasy of the data prevents both indel attraction and repulsion at least in POY. When equal gap:transformation costs are used, the resulting alignment, especially in variable regions, is longer and contains more apomorphies than alignments obtained with higher gap costs. This is because there are more gaps in the alignments. Those apomorphies, which support the strongest signal, clearly reduce the effect of indel-attraction.

Except for the prevention of indel-attraction/repulsion, the effect of homoplasious gene regions on the phylogenetic structure was not very clear. CI and RI values decreased slightly with the removal of the ITS2 data set, and the topology of trees became partially more congruent with earlier results. The effects of removing ITS2 are thus controversial, improving the results in some respects and making them worse in others. The parameter values 2:2:1 seem to suit this data better than 1:1:1, in contrast to what is the case when ITS2 data is included (Table 2).

Because the tree topologies changed comparatively little with removal of the ITS2 data set, direct optimization is clearly a very efficient way to align such highly variable regions. While trying millions of different alignments to produce certain topologies, it obviously finds the hidden signal that is congruent with the signal found in regions that are more conservative. Also, Cognato and Vogler (2001) concluded that a simultaneous POY analysis could find the common signal in data partitions, which was hidden when the partitions were analyzed one by one.

Another advantage attributed to direct optimization procedure is that alignment and tree building are not separate, consecutive steps, during which possibly different parameter values are imposed. Performing these steps separately is, according to Schulmeister et al. (2002) and Philips et al. (2000), logically inconsistent. "Without such connection, alignments might well imply other cladograms than analyses generate, or conversely, resultant cladograms might imply different alignments" (Phillips et al., 2000: 329). However, Simmons and Ochoterena (2000) consider alignment and analyses to be logically independent of each other, because in alignment hypotheses of primary homology are made and in the analyses these hypotheses are tested against each other.

TAXONOMY

The order Porellales is not resolved as a monophyletic clade with any of the parameter values used. The presence of *Marchantia polymorpha* among the Porellales in trees obtained with higher gap costs is probably due to branch attraction. Monophyly of the Porellales is further violated by the inclusion of *Ptilidium pulcherrimum* and *Radula* (Radulales). They are invariably resolved with close affinities to *Porella* and *Frullania* at the base of the ingroup. Our results lend support to Schuster's (1980a) hypothesis that *Ptilidium* might be closely related to the ancestors of the Porellinae, as well as to Schljakov's (1972) concept whereby *Radula* is a member of the Porellales. A close relationship between *Ptilidium* and Porellales is supported by the chemistry of these taxa. For example, pinguisane-type sesquiterpenes have been found in

Jungermanniales only in Ptilidiaceae, Lepidolaenaceae, Porellaceae, and Lejeuneaceae (Asakawa, 1982). However, more taxa should be included in order to test this hypothesis. It would be particularly interesting to see how the Goebeliellaceae, the monotypic family that also has been assigned to Porellales, would be placed. Below the internal taxonomy of Porellales will be discussed as it is resolved in the 1:1:1 total data tree.

The Porellales is divided in two clades, one of them containing *Ptilidium*, as a sister to *Porella*, *Radula*, and *Frullania*, and the other *Jubula* and the Lejeuneaceae (Fig. 1). The only common feature of the *Porella–Frullania–Radula* clade is the variable chromosome number in every genus of this clade. Chromosome numbers 8 and 9 have been reported in *Porella*, chromosome numbers 8, 9, 10, and 17 in *Frullania*, and 6, 8, 9, 10, 12, and 16 have been reported in *Radula* (Fritsch, 1991). In all *Jubula* and Lejeuneaceae the chromosome number is always 9.

Radula groups strongly with *Frullania*. It is difficult to find any morphological characters that support this grouping. *Radula* is unique in many features, for instance, in having its rhizoids restricted to the leaf lobules, in having often only a single oil body in the cell, in the presence of a special yellow pigment, *Radula*-type germination, and in having a dorsiventrally flattened perianth. It is also chemically very isolated (Asakawa, 1982). One explanation could be that morphological characters have evolved rapidly in *Radula* reflecting a potential adaptive radiation of this group. However, as there is no essential difference in either habitat or distribution of *Radula* compared to, for example, *Frullania*, it is hard to imagine what the adaptive pressures could have been. The relationships between *Radula* and *Frullania* need to be further studied critically.

The results strongly support the view that there should be two separate families, Frullaniaceae and Jubulaceae, rather than one, because *Frullania* and *Jubula* never compose a monophyletic group in any of the 11 trees obtained here (Fig. 3). In the total evidence 1:1:1 tree, *Frullania* and *Jubula* are even phylogenetically remote from each other (Fig. 1). Two separate families are also what Asakawa et al. (1979), Hattori (1982), and Hattori and Mizutani (1982) proposed.

Frullania tamarisci (L.) Dumort. (subg. *Frullania*) lies sister to the species from the subgenus *Trachycolea*. These subgenera have been distinguished by, among other things, different lobule shape (wider than long in *Trachycolea* and longer than wide in *Frullania*) and by that *Trachycolea* are diocious (Mizutani 1961). The node shows strong Bremer support values (Fig. 1).

The Jubulineae sensu Crandall-Stotler and Stotler (2000) containing the Frullaniaceae, Jubulaceae, and Lejeuneaceae is not resolved as a monophyletic lineage in the 1:1:1 total evidence tree. However, the Jubulaceae and the Lejeuneaceae form sister clades. This finding is congruent with the studies of Mizutani (1961), Asakawa et al. (1979), Hattori (1982), Hattori and Mizutani (1982), and Yuzawa (1991). Mizutani (1961) proposed that *Jubula* belongs to the Lejeuneaceae as the subfamily Jubuloideae. The synapomorphies for this family would be the structure of the seta (16 outer and 4 inner cells), a stylus reduced to a mere slime papilla, a maximum of two archegonia per perianth (in Lejeuneaceae only one), and the elaters attached to the capsule wall. *Jubula* also differs from the Lejeuneaceae morphologically in many respects: in having ciliate leaves, in lacking the U-shaped central cells in underleaf base, in having two archegonia per perianth instead of one, and in lacking rosette-like protrusions in spore surface. The most obvious difference is that in *Jubula* the leaf is divided to the base, so that the galeate, helmet-shaped lobule is attached to the stem only with a stalk of cells, while in the Lejeuneaceae the leaf is not divided to the base, and the leaf lobule turns upward against the ventral surface of the larger leaf lobe and is attached to the stem by many cells. Whether *Jubula* should be included in the Lejeuneaceae (or vice versa) may be a matter of taste, depending on what amount of similarity and difference one requires for either fusion or separation of families.

Several authors (Mizutani, 1985; Gradstein, 1994, 2001; Weis, 2001; Gradstein et al., 2003) consider *Nipponolejeunea pilifera* as a member of the Lejeuneaceae. Nevertheless, in the present analysis it assorts to the Jubulaceae, and this grouping can be found in all the obtained trees except the 1:1:8 tree (tree not shown). In the 1:1:1 total data tree, the Bremer support value of the node leading to this clade is high (BS = 47), when the minimal value found in the tree is 2 (Fig. 1). The position of *Nipponolejeunea* is in accordance with the results obtained by Mizutani (1961) and Ahonen et al. (2003). The morphological characters Mizutani (1961) listed to justify his inclusion of *Nipponolejeunea* into Jubuloideae were ciliate leaf lobes, narrow attachment of the ventral leaf lobule to the stem, and sharply bilobed underleaves. Also Inoue et al. (1981) found that *Nipponolejeunea* contained monoterpenes borneol and bornyl acetate,

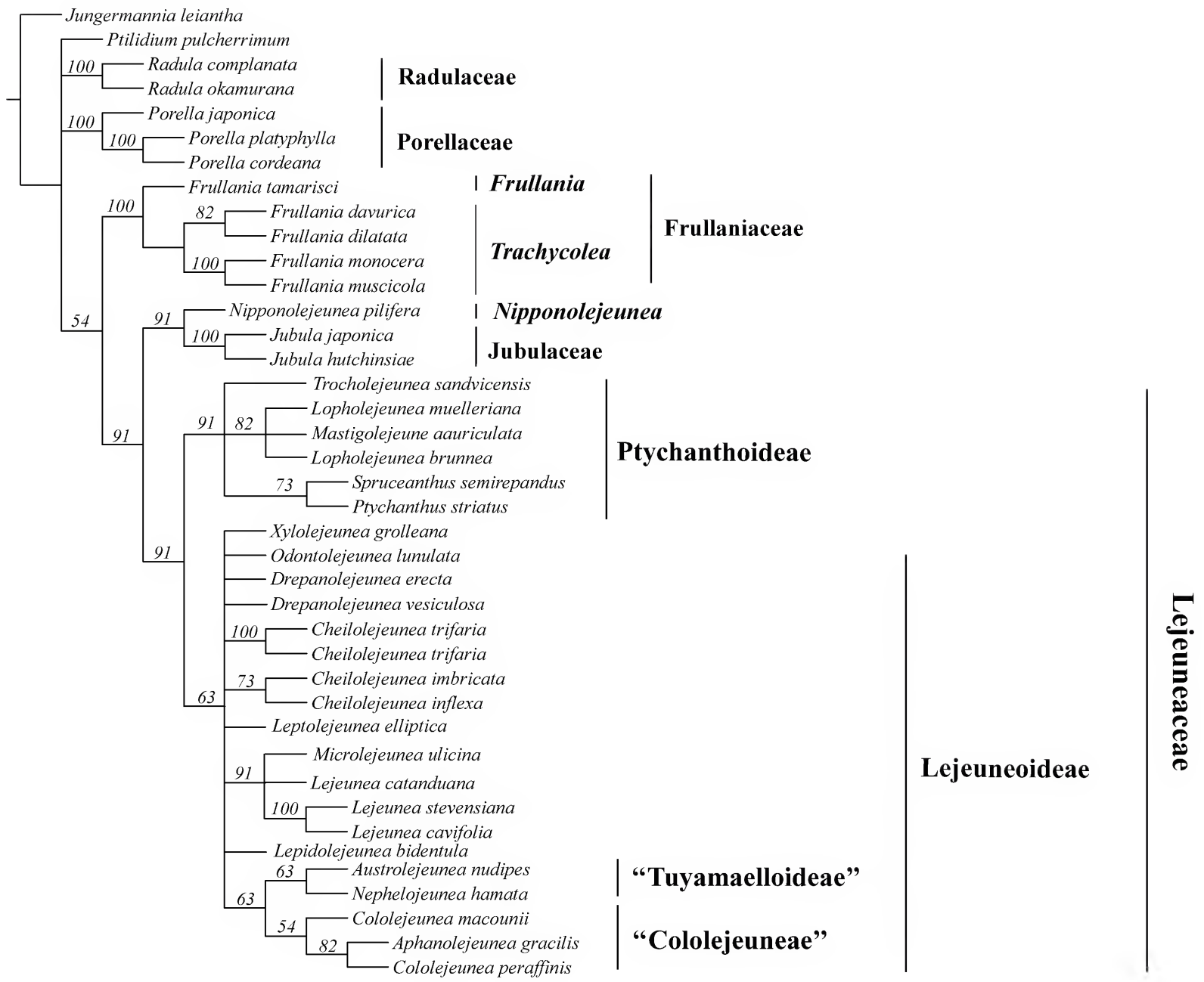

Figure 3. The majority rule consensus tree of the 17 trees obtained with different gap costs and portions of data (see Table 2). The 20 trees obtained with fixed states method were not included in the consensus tree. The numbers in the nodes are percentages of those trees, where the particular clade was found.

which are compounds not found elsewhere in the Lejeuneaceae.

Morphologically, *Nipponolejeunea* is intermediate between the Jubulaceae and the Lejeuneaceae in many respects. However, the anatomical structure of the *Nipponolejeunea* lobule is closer to that found in Lejeuneaceae, whereas the *Jubula* type lobule is shared by the Jubulaceae and the Frullaniaceae. If *Nipponolejeunea* truly belongs to the Jubulaceae, this important structure would be homoplastic. Another explanation could be the persistence of the juvenile lobule in the Lejeuneaceae and *Nipponolejeunea* because at least juvenile *Frullania* have Lejeuneaceae type lobules (Bisang, 1987).

The Lejeuneaceae (with the exception of the genus *Nipponolejeunea*) is monophyletic in the 1:1:1 tree obtained with total data. It is divided in two sister groups, the Ptychanthoideae and the Lejeuneoideae (Fig. 1). This is in accordance with Ahonen et al. (2003). In Weis (2001) and Gradstein et al. (2003), Lejeuneoideae and part of the Ptychanthoideae were sister groups, although in their studies the subfamily Ptychanthoideae as a whole appeared paraphyletic.

Odontolejeunea lunulata appears in sister position to Ptychanthoideae and Lejeuneoideae. In this study, *O. lunulata* is the sole representative of the tribe Brachiolejeuneae. Brachiolejeuneae is morphologically intermediate between Ptychanthoideae and Lejeuneoideae and has been assigned to both subfamilies: van Slageren (1985) placed it in the Ptychanthoideae and Gradstein (1987) and Kruijt (1988) in the Lejeuneoideae.

Representatives of the subfamily Ptychanthoideae form a well-supported monophyletic group in the 1:1:1 total data tree, as well as in nearly all the other trees (Fig. 3). The Ptychanthoideae has been considered more primitive than the Lejeuneoideae (e.g., Mizutani, 1961; Schuster, 1980a), but the results of the present study do not support this hypothesis. On the contrary, when the gap cost is increased, the Ptychanthoideae clade has a tenden-

cy to move upward in the tree, becoming a subgroup of Lejeuneoideae.

Trocholejeunea sandvicensis is basal in the Ptychanthoideae, while *Spruceanthus semirepandus* and *Ptychanthus striatus* form the most apical clade (Fig. 1). This is in accordance with Ahonen et al. (2003), but not with Gradstein (1994) or Gradstein et al. (2003), who have placed *Spruceanthus* and *Ptychanthus* in separate subtribes. However, *Spruceanthus* was only separated from *Ptychanthus* by Verdoorn (1934) because its perianth is 3-keeled in juvenile stage. Nevertheless, the oil bodies of *Spruceanthus* are granular, while those of *Ptychanthus* are homogenous. *Trocholejeunea sandvicensis* differs from the other Ptychanthoideae species in this study in having chromosome number in multiples of eight (nine in other species), subfloral innovations of *Frullania*-type (*Radula*-type in other species), having thin-walled medullary cells, and seta composed of more than 20 tiers of regularly arranged cells (16 outer cells and 4 inner cells in the other species) (Mizutani, 1961; Fritsch, 1991; Gradstein, 2001). All this supports the sister position of *Trocholejeunea* to the other Ptychanthoideae species in this study.

The Lejeuneoideae and Lejeuneae are monophyletic in the 1:1:1 total data tree if Cololejeuneae is accepted as a part of it. The *Drepanolejeunea* clade is sister to all the other taxa. The two *Drepanolejeunea* species group together only in trees obtained with lower gap costs (Fig. 1). The *D. vesiculosa* branch is particularly long (data not shown), indicating that molecular evolution within this family might have been faster than in most other families.

The Cololejeuneae, although monophyletic, cannot be recognized without leaving the rest of the subfamily paraphyletic (Fig. 1). This is in accordance with Weis (2001). However, in Ahonen et al. (2003), in the best-supported tree the representatives of the genus *Cololejeunea* compose a sister group to the other members of the Lejeuneoideae. The distinguishing feature of the Cololejeuneae sensu Gradstein (2001) is the retention of juvenile features in their morphology, for example, the pendular segmentation. Different aspects of this are discussed in Kis and Pócs (2001).

In Gradstein et al. (2003), Tuyamaelloideae sensu Schuster 1963b are closely related to the Cololejeuneae. Mizutani (1961) included the type genus of the Tuyamaelloideae, *Tuyamaella* S. Hatt., in the Cololejeuneoideae, because they shared many morphological features, for example, presence of seven cortical cells and narrow insertion of the leaf lobule to the stem. Schuster (1980a: 1218) stated that the "affinities of the Cololejeuneoideae are most directly with the Tuyamaelloideae, especially *Tuyamaella.* " The Tuyamaelloideae and Cololejeuneae together also form a monophyletic group in Weis (2001). In Gradstein et al. (2003), however, Cololejeuneae is paraphyletic, and only *Colura* and *Diplasiolejeunea* group with the Tuyamelloideae. In the 1:1:1 total data tree, the Tuyamaelloideae–Cololejeuneae form a sister group to all the other Lejeuneae except the basal *Drepanolejeunea* clade (Fig. 1).

Lejeunea species and *Microlejeunea ulicina* form a well-supported monophyletic group (Fig. 1). No doubt, the large and taxonomically perplexing genus *Lejeunea* would need closer studies. Mizutani (1961) suggested that *Lejeunea* would be taxonomically close to *Cheilolejeunea,* but this is not supported by the present results.

The genus *Cheilolejeunea* forms a monophyletic group in the 1:1:1 total data tree and in trees obtained with the *trn*L indel data omitted (Figs. 1, 2). *Xylolejeunea grolleana* and *Leptolejeunea elliptica* are basal in the *Cheilolejeunea* clade. *Leptolejeunea* (Spruce) Schiffn. is supposed to be closer to *Drepanolejeunea* (Mizutani, 1961), but this is not supported by the present results. *Cheilolejeunea* is a terminal clade in the Lejeuneoideae in the 1:1:1 tree obtained with total data, but a basal one in some other trees. Although the basal position appears here in less reliable trees according to the ILD values, the basal position would be supported by fossil data. Some of the earliest Lejeuneaceae fossils, ca. 40 million years old, belong to the genus *Cheilolejeunea* (Grolle, 1984). Some *Cheilolejeunea* species also have robust ventral merophytes, a feature that suggests an intermediate position between the Lejeuneoideae and Ptychanthoideae (e.g., He, 1996). However, even according to Weis (2001) and Ahonen et al. (2003), *Cheilolejeunea* was not basal in the Lejeuneoideae.

Conclusions

Taxonomy

Phylogenetic inferences from nuclear and chloroplast sequence resolve the Porellales as defined by Schuster (1958, 1963a) and by Crandall-Stotler and Stotler (2000) as a polyphyletic entity. Monophyly is restored if *Ptilidium pulcherrimum* and *Radula* are accepted as part of the order. The strong affinities of *Ptilidium pulcherrimum* to the Porellales and, in particular, the *Porella–Radula–Frullania* clade are surprising considering *P. pulcherrimum* lacks a lobule and endosporic germination. Similarly, the inclusion of *Radula* in this

clade is incongruent with previous systematic concepts because the genus shares few morphological characters with other members of the ingroup. However, both of these hypotheses are strongly supported by our data.

The Porellales are divided in two clades, which could correspond to the suborders Porellineae and Jubulineae. However, then Porellineae should include at least the Porellaceae, Frullaniaceae, Radulaceae, and *Ptilidium pulcherrimum*. *Frullania* and *Jubula* do not form a monophyletic group in any of the trees, so the results of this study give strong support to assigning them to separate families.

Nipponolejeunea pilifera is consistently resolved sister to *Jubula*. This position raises interesting questions about the evolution and developmental regulation of the lobule that should be addressed in a further study.

The remaining Lejeuneaceae compose a monophyletic lineage. The Ptychanthoideae are monophyletic and well supported (Fig. 1). There is no support for the traditional view that the Ptychanthoideae would be more basal than the Lejeuneoideae (e.g., Mizutani, 1961). Instead, the Ptychanthoideae and the Lejeuneoideae appear to be sister clades. The results do not support a division of Lejeuneoideae into tribes. *Odontolejeunea lunulata* (Lejeuneoideae, Brachiolejeuneae) is resolved as a sister to both Ptychanthoideae and Lejeuneoideae. The Brachiolejeuneae could perhaps represent the ancestral type, of which Ptychanthoideae have retained the gametophytic characters and Lejeuneoideae the sporophytic characters. The Cololejeuneae form a monophyletic lineage with the Tuyamaelloideae and are nested within the Lejeuneae, which are thereby resolved as paraphyletic.

METHODOLOGY

The strong effect of parameter choices on the tree topology was observed in the present study. Judging by the results here, the equal weighting seems to be the best alternative. It gave the best ILD values, and in addition it is the most parsimonious solution with as few ad hoc decisions as possible about frequency of base substitutions. It is also least susceptible to branch attraction. The higher the gap cost, the shorter nonhomologous deletions and insertions can draw unrelated taxa together, when gaps are treated as fifth bases.

The highly variable ITS2 region was found to prevent the branch attraction. POY aligns such regions according to the signal from unambiguous parts of the data by inserting gaps and creating synapomorphies to support this strong signal. These synapomorphies may overcome some false signals, e.g., from nonhomologous gaps. In that sense homoplasy adds phylogenetic structure, as Källersjö et al. (1999) suggested. On the other hand, just variable regions of unequal length are prone to contain nonhomologous deletions and insertions. In the light of the present results, it would be safest (although maybe not yet always possible) to use genomic regions with as few gaps as possible and not weigh those gaps higher than base substitutions.

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PHYLOGENY OF LEJEUNEACEAE: A CLADISTIC ANALYSIS OF CHLOROPLAST GENE *RBC*L SEQUENCES AND MORPHOLOGY WITH PRELIMINARY COMMENTS ON THE MITOCHONDRIAL *NAD*4–2 SPACER REGION[1]

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Abstract

Maximum likelihood (ML) analyses of a *rbc*L dataset containing 51 sequences of Lejeuneaceae (belonging to 29 genera), 4 sequences of *Jubula,* 1 sequence of *Nipponolejeunea,* 2 sequences of *Frullania,* and 1 sequence of *Porella* were performed to evaluate intergeneric relationships within the family Lejeuneaceae, and to test some generic concepts. Lejeuneaceae form a robust clade sister to the well-supported Jubulaceae including species of *Jubula* and *Nipponolejeunea. Frullania* and Jubulaceae do not form a monophyletic lineage. The division of Lejeuneaceae into the subfamilies Ptychanthoideae and Lejeuneoideae is largely supported by the molecular topology. Eighteen genera of Lejeuneoideae form a robust clade. *Microlejeunea* is placed at the base of a well-supported clade with several representatives of *Lejeunea. Macrolejeunea* is nested within *Lejeunea.* Three sequences of *Ceratolejeunea* are placed in a well-supported clade reflecting the recent division of the genus into two subgenera. The poorly known Ecuadorian endemic *Myriocolea irrorata* is placed in an unsupported sister relationship with representatives of *Cololejeunea* and *Diplasiolejeunea.* Members of nine genera of Ptychanthoideae form a monophyletic lineage, albeit without Bayesian support. *Bryopteris,* sometimes treated as a separate family Bryopteridaceae, is placed within Lejeuneaceae subfamily Ptychanthoideae. *Marchesinia* (Ptychanthoideae) forms a paraphylum with the other Ptychanthoideae and with Lejeunoideae. A well-supported sister clade to Lejeuneoideae includes *Schiffneriolejeunea* (Ptychanthoideae) and *Symbiezidium* (Lejeuneoideae). The branches of Ptychanthoideae are shorter than those of Lejeuneoideae. Maximum parsimony (MP) analyses of the *rbc*L data set in combination with a morphological data matrix led to similar topologies. Contrary to the ML topology, *Symbiezidium* is placed at the base of the robust Lejeuneoideae clade. Support for some subclades is enhanced in comparison to the ML topology. The *Myriocolea/Diplasiolejeunea/Cololejeunea* clade receives moderate bootstrap support. *Cystolejeunea* is nested within *Cheilolejeunea* and treated as a synonym of the latter. ML analysis of six sequences of the mitochondrial *nad*4–2 spacer region confirms the sister relationship of *Jubula* and *Nipponolejeunea* and representatives of Lejeuneaceae.

Key words: Lejeuneaceae, *nad*4–2, phylogeny, *rbc*L.

Lejeuneaceae are the largest family of liverworts with about 1000 species in 90 currently accepted genera (Gradstein et al., 2003). The center of diversity is the humid tropics where Lejeuneaceae grow mainly as epiphytes on bark and living leaves. Especially in lowland forests, Lejeuneaceae often dominate the cryptogamic flora and may represent more than 70 percent of the species inventory (Gradstein, 1994).

The family is characterized by incubous leaves with a small ventral lobule attached along a keel, the presence of underleaves and/or rhizoid tufts, and the existence of only one archegonium per perianth. A wide range of morphological variation is seen across the family. However, the supraspecific division of Lejeuneaceae is hampered by the scarcity of stable morphological characters. Attempts to classify the family based on morphology

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have been numerous and have often led to controversial results (e.g., Mizutani, 1961; Schuster, 1963, 1966, 1992, 2001; Grolle, 1964, 1983; Gradstein, 1975, 1994; Gradstein et al., 2001, 2003). At least nine subfamilies and many tribes and subtribes have been recognized in the past (compare Schuster, 1963; Gradstein, 1994; Weis, 2001; Gradstein et al., 2003).

Only a few morphological studies of Lejeuneaceae have included cladistic analyses (Gradstein, 1994; Weis, 2001). Gradstein et al. (2003) analyzed a data matrix with 49 generic characters of sporophytes and gametophytes and one chemical character. The strict consensus tree obtained from this matrix was largely unresolved; nevertheless, the study supported the recognition of two broad subfamilies, Ptychanthoideae Mizut. and Lejeuneoideae.

Ahonen et al. (2003) published the first molecular phylogenetic analysis of 12 genera of Lejeuneaceae using partial cp *rbc*L and *trn*L-*trn*F as well as nr ITS2 sequences. The division of Lejeuneaceae into the two previously mentioned subfamilies was supported by their analyses. Another important result of their study was the exclusion of *Nipponolejeunea* S. Hatt. from Lejeuneaceae. Groth-Malonek et al. (2004) analyzed relationships among nine genera of Lejeuneaceae using sequences of the entire nr ITS region. They found that deeper nodes were not supported but several robust generic and tribal clades did receive significant bootstrap support. The data also indicated that *Bryopteris* (Nees) Lindenb., sometimes treated as a separate family Bryopteridaceae (e.g., Crandall-Stotler & Stotler, 2000), is a member of Lejeuneaceae subfamily Ptychanthoideae.

In recent years, the chloroplast gene marker *rbc*L has proven to be a powerful source of signal to infer the phylogeny of mosses (e.g., Goffinet et al., 1998; Arikawa & Higuchi, 1999; Tsubota et al., 2001, 2002; Akiyama et al., 2003) and to investigate the main lineages of hepatics (Lewis et al., 1998). In this paper we utilize *rbc*L sequences from a wide taxon sampling to evaluate intergeneric relationships within the family Lejeuneaceae and to test generic concepts.

Slow-evolving mitochondrial DNA have been used in the past to infer the phylogeny of bryophytes above family level (e.g., Nickrent, 2000; Beckert et al., 2001; Pruchner et al., 2002). Here we use the *nad*4–2 spacer region as well as parts of the surrounding genes to assess its suitability as a further possible marker for Lejeuneaceae.

Materials and Methods

Taxon sampling and designation of the outgroup

Sequences from a total of 61 accessions were used for this study (Table 1), including 18 *rbc*L sequences from Ahonen et al. (2003) and 1 *rbc*L sequence from Lewis et al. (1998). Vouchers related to new sequences are deposited at the herbarium of the University of Göttingen (GOET), with the exception of *Nipponolejeunea subalpina* (Horikawa) S. Hatt., for which the voucher is kept at the Hiroshima University Herbarium (HIRO).

Ingroup species of the two Lejeuneaceae subfamilies Ptychanthoideae and Lejeuneoideae were selected based on available material and to represent the morphological variation of the family. The sampling included 28 genera of Lejeuneaceae, several of which are represented by more than one sequence. The ingroup was completed with sequences of *Nipponolejeunea, Jubula* Dumort., and *Frullania* Raddi. *Porella* L. (*rbc*L data set) and *Plagiochila* (Dumort.) Dumort. (*nad*4–2 spacer data set) were designated as outgroups based on phylogenetic analyses of a larger dataset of bryophyte *rbc*L sequences from Lewis et al. (1998) and own unpublished sequences (data not shown).

DNA extraction

Plant tissue from the distal portions of a few shoots was selected from up to ca. 15-year-old herbarium specimens. Total genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany).

PCR amplification and sequencing

The polymerase chain reaction (PCR) was performed in a total volume of 50 µl containing 1.5 µl template DNA (ca. 50 ng), 1 U SilverStar *Taq* Polymerase (EuroGenTech), 5 µl 10× PCR buffer for SilverStar *Taq* Pol, 1 µl dNTP mix (10 mM), 2 µl $MgCl_2$ (50 mM), 2 µl DMSO, 1 µl upstream primer (10 µM), 1 µl downstream primer (10 µM), and 36.5 µl double distilled water.

The *rbc*L PCR program was as follows: 2 min. initial denaturation at 92°C, followed by 25 cycles of 1 min. denaturation at 92°C, 50 sec. annealing at 51°C, and 1.5 min. elongation at 72°C. Final elongation was carried out in one step of 10 min. at 72°C. The primers *rbc*L-1Pl-F and M-1390R (Table 2) were used for the first PCR. If no product could be detected (on 1% agarose gel stained with GelStar [MFC BioProducts, Rockland, Maine, U.S.A.]), then a second nested PCR was carried out

with the primers *rbc*L-38F and M-1379R. Sequencing primers were *rbc*L-38F, *rbc*L-680Pl-F, *rbc*L-700Pl-R, and *rbc*L-1200-R. The primers *rbc*L-1Pl-F, M-1379R, and M-1390R were taken from the research web page of Wolf (⟨http://bioweb.usu.edu/wolf/rbcL%20primer%20map.htm⟩). *Rbc*L-38F, *rbc*L-680Pl-F, *rbc*L-700Pl-R, and *rbc*L-1200-R were designed for this study (Table 2).

The *nad*4–2 spacer PCR program included an initial denaturation of 1 min. at 92°C, followed by 9 cycles with 1 min. of denaturation at 92°C, 1 min. of annealing at 57°C minus 0.7°C per cycle, and 1 min. of synthesis at 72°C. This was followed by a further 29 cycles that included a further step of denaturation for 1 min. at 92°C, 1 min. annealing at 50°C followed by 2 min. of synthesis at 72°C plus 1 sec. per cycle. Final elongation was carried out in one step of 15 min. at 72°C. PCR primers for the *nad*4–2 spacer were *nad*4RB–1F and *nad*2LB–1R (Groth-Malonek, unpublished; Table 2). The sequencing primer was *nad*2LB–1R.

Sequencing was carried out on an ABI 3100 capillary sequencer using the BigDye Terminator Cycle Sequencing v2.0 kit (PE Applied Biosystems, Foster City, California, U.S.A.).

PHYLOGENETIC ANALYSES

The 59 *rbc*L sequences and six *nad*4–2 spacer region sequences were aligned manually in BioEdit version 5.0.9 (Hall, 1999), resulting in an *rbc*L alignment with 1301 sites and a *nad*4–2 spacer region alignment with 183 sites. The lacking parts of several *rbc*L sequences were scored as missing. The *rbc*L data set was analyzed separately and in combination with a morphological data matrix of 49 gametophytic or sporophytic generic characters and one chemical character taken from Gradstein et al. (2003). The alignments are available from RW upon request. Phylogenetic trees were inferred using maximum likelihood (ML) and maximum parsimony (MP) criteria as implemented in PAUP* version 4.0b10 (Swofford, 2000).

ML analyses. To decide on the nucleotide substitution model with the smallest number of parameters that best fits the data, the program Modeltest 3.06 (Posada & Crandall, 1998) was used. Based on the results of the tests, the model selected by the hierarchical likelihood ratio tests for both the *rbc*L and the *nad*4–2 spacer region data set was TrN (Tamura & Nei, 1993) with proportion of invariable sites (I) and gamma shape parameter (G) for among site variation calculated from the dataset (TrN + I + G). The confidence of branching of the *rbc*L tree was assessed using MrBayes (version 3.0b4; Huelsenbeck & Ronquist, 2001), a program for Bayesian inference of phylogeny. The Modeltest 3.06 output values were applied to the command block of MrBayes. Five hundred thousand generations were calculated, of which every 100 was saved, resulting in 5000 saved trees. Trees collected before the likelihood score had stabilized were deleted. The remaining 3500 trees with a stable likelihood score were condensed to a majority rule consensus tree. Branch support of the consensus tree, referred to as posterior probabilities, was regarded as significant when exceeding 0.95 (Larget & Simon, 1999). The confidence of branching of the *nad*4–2 spacer region tree was assessed using 1000 bootstrap resamplings in ML analysis (using the TrN + I + G model, heuristic search with 10 addition sequence replicates).

MP analyses. Maximum parsimony analyses of the combined *rbc*L–morphology data set were performed with the following options implemented: heuristic search mode with 1000 random-addition-sequence replicates, tree bisection-reconnection branch swapping (TBR), MulTrees option on, and collapse zero-length branches off. All characters were treated as equally weighted and unordered. Non-parametric bootstrap support (Felsenstein, 1985) was estimated with full heuristic search, 1000 bootstrap replicates, and 10 random addition sequence replicates per bootstrap replicate, TBR swapping, MulTrees option on, collapse zero-branches off, and by saving all trees. Bootstrap percentage values between 70 and 100% were regarded as significant branch support.

RESULTS

*RBC*L MAXIMUM LIKELIHOOD TREE

The maximum likelihood analysis produced three most likely trees (−ln = 7610.7608) that differed only in minor discrepancies in branch lengths, and hence, only one tree is depicted here (Fig. 1). The representatives of Lejeuneaceae are placed in a well-supported clade sister to the robust Jubulaceae clade, which includes species of *Jubula* and *Nipponolejeunea.* Compared to the branch lengths within the Lejeuneaceae and the Jubulaceae clades, the two families are separated by a relatively large genetic distance. Two species of *Frullania* form a robust clade sister to that containing Lejeuneaceae and Jubulaceae. Sequences from 14 genera of Lejeuneaceae subfamily Lejeuneoideae are placed within a robust clade (Bayesian support = 1.0). Several subclades are well resolved. A robust clade includes members of the genera *Lejeunea* Lib. s.l. (*Macrolejeunea* (Spruce)

Table 1. Voucher list. GenBank accession numbers in bold are new for this study; those in italics are from Lewis et al. (1998); others are from Ahonen et al. (2003). All vouchers of sequences produced for this study are deposited at GOET, with the exception of *Nipponolejeunea subalpina*, which is at HIRO.

Taxon	Origin	Voucher	*rbc*L	*nad4-2 spacer region*
Archilejeunea planiuscula (Mitt.) Steph.	Thailand	*Schäfer-Verwimp 16237*	**AY548081**	
Arcolejeunea fertilis (Reinw. Blume & Nees) Spruce ex Steph.	Bali	*Schäfer-Verwimp 17009*	**AY684929**	
Aureolejeunea aurifera R. M. Schust.	Costa Rica	*Holz CR00-696*	**AY548082**	
Aureolejeunea fulva R. M. Schust.	Brazil	*Costa & Gradstein 3849*	**AY548083**	
Bryopteris diffusa (Sw.) Nees	Bolivia	*Gradstein 9929*	**AY548084**	
Bryopteris diffusa	Bolivia	*Acebey 855*	**AY548085**	
Bryopteris filicina (Sw.) Nees	Bolivia	*Heinrichs & Müller 4031*	**AY548086**	
Bryopteris filicina	Bolivia	*Drehwald 4751*	**AY548087**	
Ceratolejeunea cornuta (Lindenb.) Steph.	Bolivia	*Drehwald 4739*	**AY548088**	
Ceratolejeunea filaria (Taylor ex Lehm.) Steph.	Ecuador	*Dauphin 2700*	**AY548089**	**AY548060**
Ceratolejeunea grandiloba J. B. Jack & Steph.	Bolivia	*Gradstein 7055*	**AY548090**	
Cheilolejeunea sp. indet.	Honduras	*Allen 17393*	**AY548091**	
Cheilolejeunea imbricata (Nees) S. Hatt.	China	*Koponen et al. 51583*	AY125935	
Cheilolejeunea lineata (Lehm. & Lindenb.) Schiffn.	Guadeloupe	*Schäfer-Verwimp 22183*	**AY548092**	
Cheilolejeunea trifaria (Reinw., Blume & Nees) Mizut.	Bolivia	*Gradstein 9951A*	**AY548093**	
Cheilolejeunea trifaria	Bolivia	*Gradstein 9951B*	**AY548094**	
Cololejeunea macounii (Spruce) A. Evans	China	*Koponen et al. 56319*	AY125942	
Cololejeunea peculiaris (Herzog) Benedix	Malaysia	*Schäfer-Verwimp 18861/A*	**AY548095**	
Cololejeunea peraffinis (Schiffn.) Schiffn.	China	*Koponen et al. 57141*	AY125941	
Cyclolejeunea peruviana (Lehm. & Lindenb.) A. Evans	Colombia	*Gradstein 8546*		**AY548061**
Diplasiolejeunea involuta S. Winkl.	Ecuador	*Schäfer-Verwimp 23508*	**AY548096**	
Drepanolejeunea biocellata A. Evans	Ecuador	*Gradstein 10053*	**AY548097**	
Drepanolejeunea erecta (Steph.) Mizut.	China	*Koponen et al. 50605*	AY125940	
Frullania dilatata (L.) Dumort.	Finland	*Ahonen & Engblom 15*	AY125929	
Frullania holostipula S. Hatt. & D. G. Griffin	Venezuela	*Pócs 9736/CA*	**AY548098**	
Frullanoides densifolia Raddi	Bolivia	*Gradstein 9949*	AY548099	
Jubula bogotensis Steph.	Mexico	*Gradstein s.n.*	**AY548100**	**AY548062**
Jubula hutchinsiae (Hook.) Dumort.	Portugal	*Drehwald 3007*	**AY548101**	
Jubula hutchinsiae	Taiwan	*Ahonen et al. 3190*	AY125946	
Jubula japonica Steph.	China	*Koponen et al. 54308*	AY125938	
Lejeunea catanduana (Steph.) H. A. Mill., Bonner & Bischl.	China	*Koponen et al. 56008*	AY125943	
Lejeunea cavifolia (Ehrh.) Lindb.	Germany	*Heinrichs 3695*	**AY548102**	

Table 1. Continued.

Taxon	Origin	Voucher	*rbc*L	*nad4-2 spacer region*
Lejeunea cavifolia	Finland	*Ahonen s.n.*	AY125945	
Lejeunea laetevirens Nees & Mont.	Dominica	*Schäfer-Verwimp 17899*	**AY548103**	
Lejeunea mimula Hürl.	Bali	*Schäfer-Verwimp 20930*	**AY548104**	
Lepidolejeunea bidentula (Steph.) R. M. Schust.	China	*Koponen et al. 51525*	AY125936	
Lepidolejeunea eluta (Nees) R. M. Schust.	Bolivia	*Churchill & Vasquez 2180*	**AY548066**	
Leptolejeunea elliptica (Lehm. & Lindenb.) Schiffn.	China	*Koponen et al. 50179*	AY125939	
Lopholejeunea brunnea Horik.	China	*Koponen et al. 51570*	AY125930	
Lopholejeunea eulopha (Taylor) Schiffn.	Australia	*Pócs & Streimann 9987/H*	**AY548067**	
Macrolejeunea pallescens (Mitt.) Steph.	Ecuador	*Schäfer-Verwimp 23533*	**AY548068**	
Marchesinia brachiata (Sw.) Schiffn.	Ecuador	*Gradstein et al. 10052*	**AY548069**	
Mastigolejeunea auriculata (Wilson & Hook.) Schiffn.	Bolivia	*Churchill 21275*	**AY548070**	
Mastigolejeunea auriculata	Bali	*Schäfer-Verwimp 20780*	**AY548071**	
Mastigolejeunea auriculata	China	*Koponen et al. 51516*	AY125933	
Microlejeunea ulicina (Taylor) A. Evans	China	*Koponen et al. 495531*	AY125944	
Myriocolea irrorata Spruce	Ecuador	*Gradstein et al. 10033A*	**AY548072**	**AY548063**
Myriocolea irrorata	Ecuador	*Gradstein et al. 10033B*	**AY548073**	
Nipponolejeunea pilifera (Steph.) S. Hatt.	Japan	*Ohniishi s.n.*	AY125937	
Nipponolejeunea subalpina (Horik.) S. Hatt.	Japan	*Ohnishi 5611*		**AY548064**
Omphalanthus filiformis (Sw.) Nees	Bolivia	*Drehwald 4462*	**AY548074**	
Plagiochila rutilans Lindenb.	Bolivia	*Groth 101*		**AY548065**
Porella pinnata L.	U.S.A.	*Mishler et al. 3773*	*U87088*	
Ptychanthus striatus (Lehm. & Lindenb.) Nees	China	*Koponen et al. 57218*	AY125931	
Pycnolejeunea densistipula (Lehm. & Lindenb.) Steph.	Ecuador	*Schäfer-Verwimp 23368*	**AY548075**	
Schiffneriolejeunea pappeana (Nees) Gradst.	Réunion	*Schäfer-Verwimp 20314*	**AY548076**	
Schiffneriolejeunea tumida (Nees) Gradst.	Bali	*Schäfer-Verwimp 20952*	**AY548077**	
Spruceanthus semirepandus (Nees) Verd.	China	*Koponen et al. 50971*	AY125932	
Spruceanthus theobromae (Spruce) Gradst.	Ecuador	*Anderson & Kautz 1820*	**AY548078**	
Symbiezidium transversale (Sw.) (Trevis.)	Bolivia	*Gradstein 9924*	**AY548079**	
Thysananthus amazonicus (Spruce) Schiffn.	Suriname	*Muñoz 98-19*	**AY548080**	
Trocholejeunea sandvicensis (Gottsche) Mizut.	China	*Koponen et al. 55768*	AY125934	

Table 2. Primers.

Primer name	Direction	Sequence (5′–3′)	Reference
***rbc*L**			
*rbc*L-1Pl-F	Forward	ATG TCA CCA CAA ACG GAG ACT AAA RCA GGT	changed after Wolf*
*rbc*L-38-F	Forward	GGT GTT GGA TTT AAA GCT GGT G	new
*rbc*L-680Pl-F	Forward	GCY GAA ACT GGT GAA ATT AAA G	new
*rbc*L-700Pl-R	Reverse	GTC CTT TAA TTT CAC CAG TTT C	new
*rbc*L-1200-R	Reverse	TGY CCY AAA GTT CCA CCA CC	new
*rbc*L-1379-R	Reverse	TCA CAA GCA GCA GCT AGT TCA GGA CTC	changed after Wolf*
M1390-R	Reverse	CTT TCC AWA YTT CRC AAG CAG CRG	changed after Wolf*
***nad*4-2spacer region**			
*nad*4RightBorder-1F	Forward	GGA GTT ATT TGG ATG GGT GTT TAC	new, designed by Groth-Malonek
*nad*2LeftBorder-1R	Reverse	AAC ACT AAG TAA ACC AAG CCA ACY CAC	new, designed by Groth-Malonek

* Taken from the research web site of Paul G. Wolf at Utah State University, Logan, Utah, U.S.A. ⟨http://bioweb.usu.edu/wolf/rbcL%20primer%20map.htm⟩

Schiffn., *Microlejeunea* Steph., and *Lejeunea*), *Cololejeunea* (Spruce) Schiffn., *Diplasiolejeunea* (Spruce) Schiffn., and *Myriocolea* Spruce. Representatives of *Lejeunea* from tropical Asia, Europe, and the Neotropics form a well-supported monophyletic lineage. *Macrolejeunea* is nested within *Lejeunea. Microlejeunea* is sister to *Lejeunea. Diplasiolejeunea, Myriocolea,* and *Cololejeunea* form a non-supported clade sister to the *Lejeunea–Microlejeunea* clade. The monophyly of *Cololejeunea* (represented by three species) is well supported. *Lepidolejeunea* R. M. Schust., *Ceratolejeunea* J. B. Jack & Steph., *Drepanolejeuna* (Spruce) Schiffn., and *Leptolejeunea* (Spruce) Schiffn. form a paraphyletic grade at the base of the *Lejeunea–Cololejeunea* clade. The monophyly of *Lepidolejeunea, Ceratolejeunea,* and *Drepanolejeuna* (each comprising two or three species) is well supported. *Ceratolejeunea grandiloba* J. B. Jack & Steph. is placed sister to *C. cornuta* (Lindenb.) Steph. and *C. filaria* (Lehm.) Steph. *Aureolejeunea* R. M. Schust., *Omphalanthus* Lindenb. & Nees, and *Cheilolejeunea* (Spruce) Schiffn. are placed in a robust clade sister to *Pycnolejeunea* (Spruce) Schiffn. *Cheilolejeunea* is resolved paraphyletic with *Cheilolejeunea trifaria* (Reinw., Blume & Nees) Mizut. placed sister to *Omphalanthus* and a further three species of *Cheilolejeunea* in a separate clade. *Cheilolejeunea lineata* (Lehm. & Lindenb.) Schiffn., previously regarded as the monospecific genus *Cystolejeunea* A. Evans (Evans, 1906), is nested within this smaller *Cheilolejeunea* clade.

Symbiezidium transversale (Sw.) Trevis. (Lejeuneoideae) and two species of *Schiffneriolejeunea* Verd. (Ptychanthoideae) form a well-supported clade sister to the above Lejeuneoideae clade. Representatives of a further 10 genera of Ptychanthoideae form a largely unresolved paraphyletic grade at the base of the Lejeuneaceae clade with *Marchesinia* Gray as the basalmost genus. The genus *Bryopteris* is nested within Ptychanthoideae. The monophyly of *Bryopteris diffusa* (Sw.) Nees, *B. filicina* (Sw.) Nees, and *Mastigolejeunea auriculata* (Wilson & Hook.) Schiffn., each represented by two or three sequences, receive good Bayesian support. *Frullanoides* Raddi is nested within *Bryopteris* albeit without support. *Spruceanthus semirepandus* (Nees) Verd. is placed sister to *Thysananthus amazonicus* (Spruce) Schiffn., whereas *Spruceanthus theobromae* (Spruce) Gradst. is sister to *Archilejeunea planiuscula* (Mitt.) Steph. Average branch lengths are greater in Lejeuneoideae than in Ptychanthoideae.

*RBC*L–MORPHOLOGY MAXIMUM PARSIMONY TREE

Of the 1351 investigated characters (1301 molecular and 50 morphological), 259 molecular and all morphological characters were parsimony informative. The strict consensus tree of the six most parsimonious trees from the combined morphology–*rbc*L data set (Fig. 2) reveals a similar topology to that of the ML tree from the *rbc*L data set. In contrast to the ML tree, *Symbiezidium transversale* is placed at the base of the Lejeuneoideae clade in the MP tree. *Schiffneriolejeunea* is placed sister to Lejeuneoideae. The remaining investigated genera of Ptychanthoideae form a monophyletic clade sister to Lejeuneoideae and *Schiffneriolejeunea.*

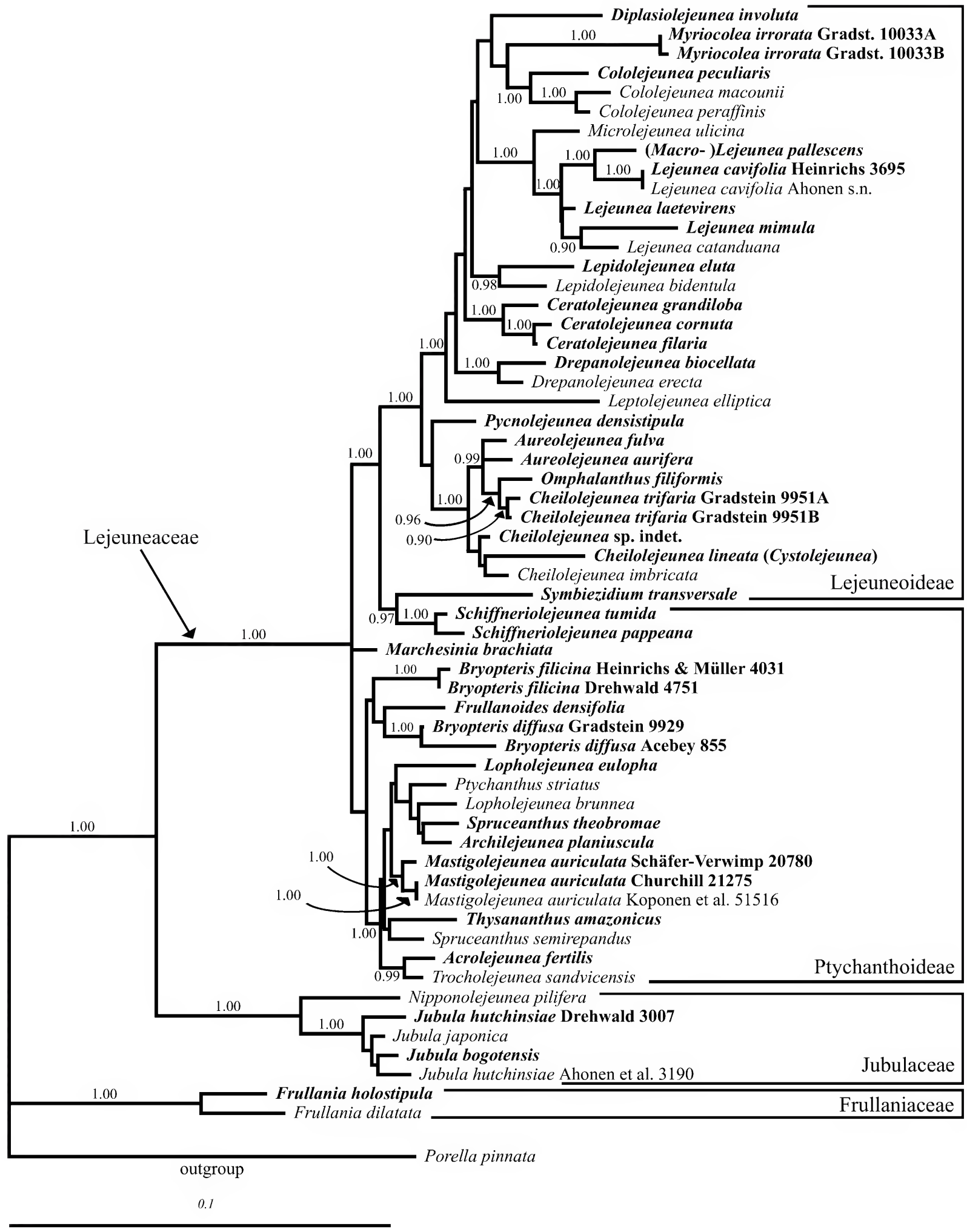

Figure 1. Molecular phylogeny of Lejeuneaceae based on *rbc*L sequence comparisons using 1301 aligned positions. One of the three most likely trees (−ln = 7610.7608) that resulted from a maximum likelihood analysis, using the TrN model with estimated gamma shape (G = 0.8791) and proportion of invariable sites (I = 0.31), calculated as the best model by Modeltest 3.06. Bayesian support (> 0.90) is indicated above the branches. Sequences produced in Göttingen are shown in bold. Other sequences from Ahonen et al. (2003) and Lewis et al. (1998).

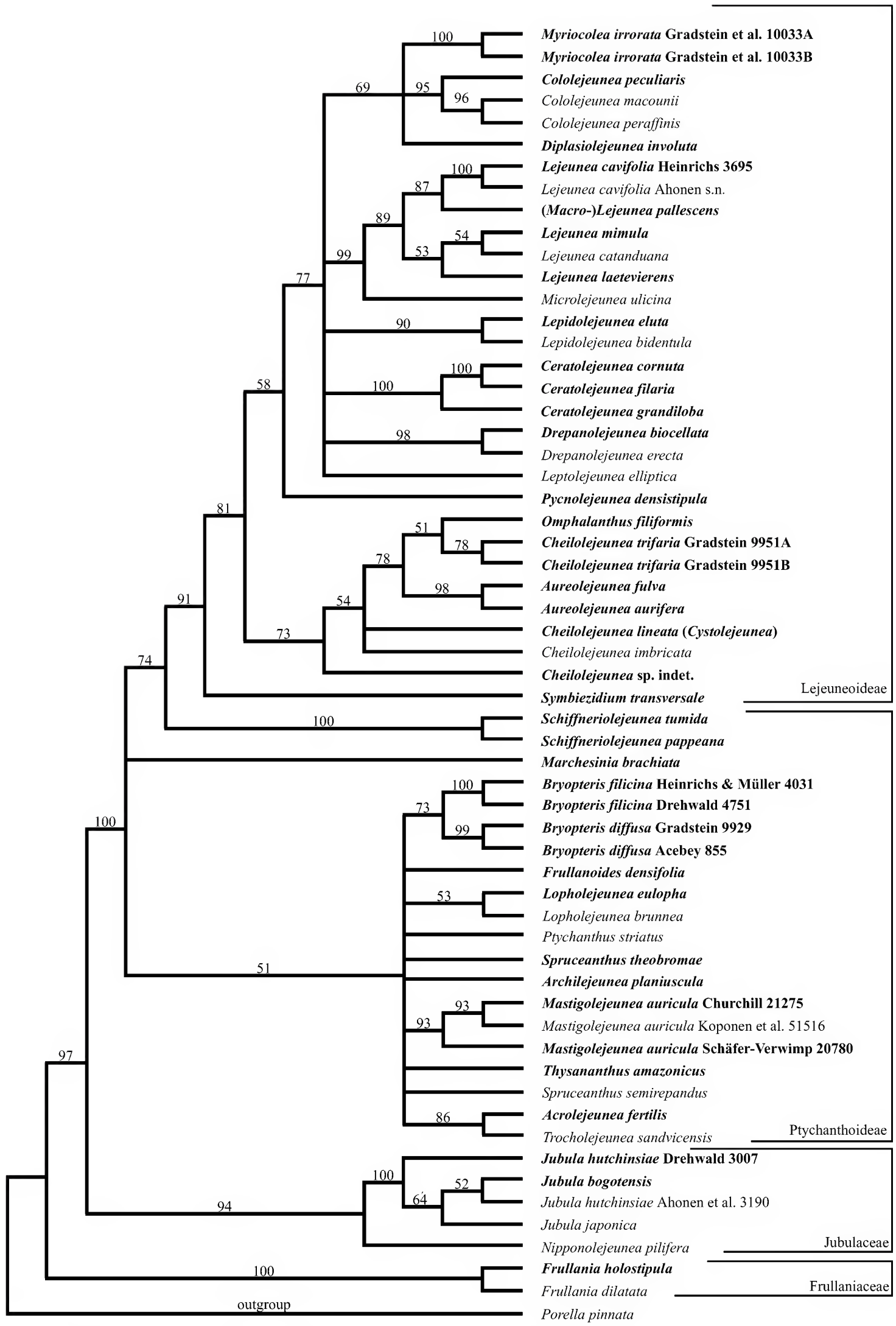

Figure 2. Rooted strict consensus of 6 equally parsimonious trees recovered during 1000 random taxon addition heuristic searches of the combined *rbc*L–morphology data sets. Bootstrap support (> 50%) is indicated above branches. Sequences produced in Göttingen are shown in bold. Other sequences from Ahonen et al. (2003) and Lewis et al. (1998).

*NAD*4–2 SPACER REGION MAXIMUM LIKELIHOOD TREE

All six investigated species have a spacer of 26 base pairs in length, of which one bp is variable. Of the 94 bp investigated of the *nad*4 gene, six are variable, while two variable positions were detected in the 63 bp of the *nad*2 gene investigated. Maximum likelihood analysis of the alignment led to a similar topology to that revealed from the *rbc*L data set (Fig. 3). Lejeuneaceae represented by *Myriocolea*, *Ceratolejeunea*, and *Cyclolejeunea* A. Evans are placed sister to a Jubulaceae clade with *Nipponolejeunea* and *Jubula*.

DISCUSSION

1. THE *RBC*L TREES

Phylogenetic inferences based on *rbc*L sequences yield a relatively well-resolved topology with a robust sister relationship of Lejeuneaceae and Jubulaceae. The Jubulaceae clade is here represented by four species of *Jubula* and one of *Nipponolejeunea*. *Nipponolejeunea* is an Asian genus characterized by leaves with long cilia, very short leaf insertion, one or two archegonia per perianth, and a capsule wall containing red alar cells and a homogeneous thickening layer on the inner surface. Several authors (e.g., Weis, 2001; Gradstein et al., 2003) have placed *Nipponolejeunea* at the base of Lejeuneaceae, while others (e.g., Mizutani, 1961) have suggested that it is better placed as sister to *Jubula* due to several characters common to both genera, which are otherwise unknown in Lejeuneaceae (e.g., red alar cells in capsule wall and one or two archegonia per perianth). Both MP and ML analyses support the placement of *Nipponolejeunea* as sister to *Jubula*, as also recently shown by Ahonen et al. (2003). By excluding *Nipponolejeunea* from Lejeuneaceae, however, one of the characters used to define Lejeuneaceae (leaves with a lobule attached to the lobe along a long keel) becomes invalid. Despite the diffuse morphology, the two families are clearly separated in the *rbc*L topology. The ML tree gives some evidence for a sudden radiation of Lejeuneaceae since the basal branches within the family are short and build a largely polytomous topology. Schuster (1963, 1980) assumed a sudden explosive radiation, which he attributed to the coevolution of Lejeuneaceae with the radiation of the angiosperms and the associated increase in available habitats. In recent years, *Frullania* has been included in Jubulaceae, leading to a synonymy of Frullaniaceae and Jubulaceae (e.g., Gradstein et al., 2001). As an outcome of our analyses, *Frullania* is recovered as not forming a monophyletic lineage with Jubulaceae. However, the taxon sampling presented here is very limited, and it is therefore too soon to make any definite statements about the relationship between Jubulaceae and Frullaniaceae.

The division of Lejeuneaceae into the two broad subfamilies Lejeuneoideae and Ptychanthoideae is largely supported by the *rbc*L topologies with better resolution seen within the subfamily Lejeuneoideae than Ptychanthoideae. This is a result of the greater number of variable positions within Lejeuneoideae as indicated by the longer branches seen within the subfamily. Lejeuneoideae contain many of the smaller epiphyllous members of the family, which are perhaps characterized by a more opportunistic mode of life and fast adaptation to any new habitats and ecological niches that became available (Thiers, 1988; Gradstein, 1997).

Some noteworthy incongruences between morphological concepts and the *rbc*L topologies must also be discussed. The subfamily Ptychanthoideae are morphologically characterized by undivided underleaves, capsule valves wide-spreading after dehiscence, a rectangular hinge on outer valve surface that extends to valve margins, an inner valve surface with a fenestrate thickening layer, elaters with upper ends fixed at valve surface and margins, and tetrahedrical spore tetrads. The subfamily Lejeuneoideae are characterized by undivided or bifid underleaves, capsule valves not wide-spreading after dehiscence, the hinge either horseshoe-, butterfly- or quadrate-shaped and not extending to valve margins, an inner valve surface without fenestrate thickening layer, elaters with upper ends fixed at valve margins only, and decussate spore tetrads (Gradstein, 1994; Gradstein et al., 2003). However, the division into these morphologically well-separated groups is not completely reflected in the *rbc*L topologies. The large Lejeuneaceae clade is divided into three smaller clades: the two classically accepted subfamilies (Ptychanthoideae and Lejeuneoideae), and a smaller third clade comprising two *Schiffneriolejeunea* species and (only seen in the ML tree) *Symbiezidium transversale*. Morphologically, *Schiffneriolejeunea* is clearly a member of Ptychanthoideae, and its position here sister to Lejeuneoideae is not supported by any morphological evidence (Gradstein, 1994). The division of *Schiffneriolejeunea* from the rest of Ptychanthoideae could be an indication of a further Ptychanthoideae clade but due to the relatively sparse taxon sampling this can not be determined at this stage. *Symbiezidium transversale* is morphologically classified as a member of Brachiolejeuneeae, a tribe within

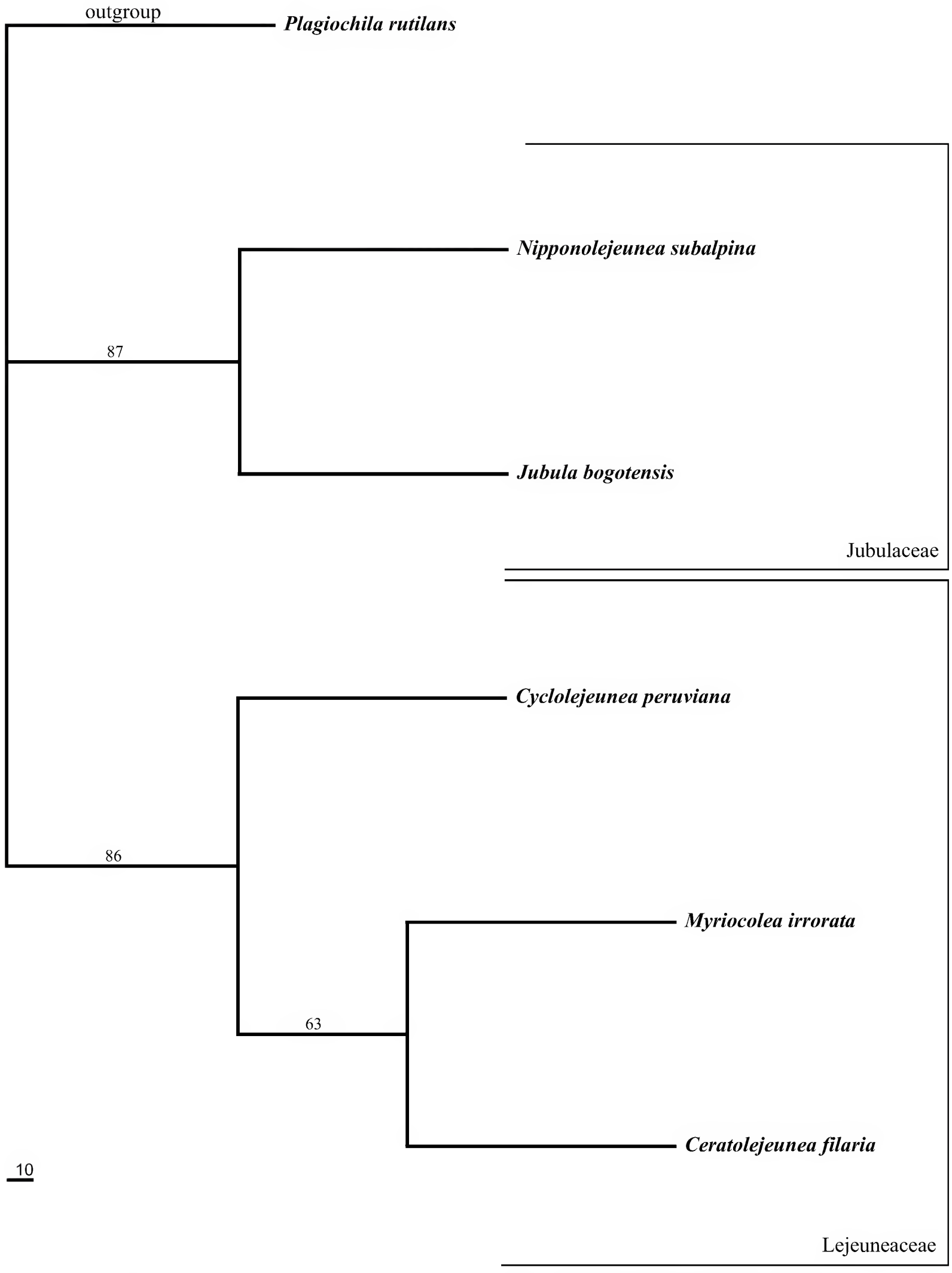

Figure 3. Molecular phylogeny of Lejeuneaceae based on the *nad*4–2 spacer region (181 positions). Single most likely tree (−ln = 323.425) that resulted from a maximum likelihood analysis, using the TrN model with estimated gamma shape (G = 0.8791) and proportion of invariable sites (I = 0.31), calculated as the best model by Modeltest 3.06. Bootstrap support (> 70%) is indicated above branches.

Lejeuneoideae (Gradstein, 1994). Its position sister to *Schiffneriolejeunea* within this third robust clade (ML analysis) may be influenced by the incomplete taxon sampling, which lacks further representatives of the Brachiolejeuneeae. The MP analysis of the combined molecular and morphological matrix, however, placed *Symbiezidium* at the base of the Lejeuneoideae, as would be expected from morphological study. The data clearly indicate that further work with more specimens is needed to clarify the systematic positions of both *Schiffneriolejeunea* and *Symbiezidium*. An alternative interpretation of the *rbc*L topology is that Lejeuneoideae have evolved from Ptychanthoideae and that morphological evolution is not absolutely congruent with the evolution of *rbc*L.

The systematic position of Bryopteris

Stotler and Crandall-Stotler (1974) and Crandall-Stotler and Stotler (2000) have argued that *Bryopteris* should be placed in its own family (Bryopteridaceae) on grounds of its unusual branching. In contrast, our results show it to be placed within the subfamily Ptychanthoideae, which is in agreement with other authors (e.g., Gradstein, 1994; Weis, 2001; Gradstein et al., 2003; Groth-Malonek et al., 2004). Although both species clades (*Bryopteris filicina* and *B. diffusa*) receive significant support in both the MP and ML analyses, good support for the entire *Bryopteris* clade is only seen in the MP tree of the combined morphology–*rbc*L data set. However, the incorporation of Bryopteridaceae in Lejeuneaceae is also supported by ITS trees (Groth-Malonek et al., 2004). Therefore, we see no reason why *Bryopteris* should not remain a member of Lejeuneaceae. Noteworthy is also that *Bryopteris filicina* and *B. diffusa* are placed in an unsupported clade together with *Frullanoides densifolia* Raddi (ML analysis). The two *Bryopteris* species are known to be quite different from another morphologically (Gradstein, 1994), and further work with ITS should help to clarify the phylogenetic relationships of species within the *Bryopteris–Frullanoides* clade. Work to gain these data is already underway.

Spruceanthus theobromae

Spruceanthus theobromae, the only Neotropical species in an otherwise Asian genus, is known to differ in leaf cell shape from other members of the genus (Gradstein, 1994, 1999). Although there is little support to confirm its placement, *Spruceanthus theobromae* is not placed together with *Spruceanthus semirepandus*, the type species of the genus *Spruceanthus*, in the ML tree. These two species are indeed positioned in the same clade in the MP analysis, but without any support, and this is probably an artefact of the morphological matrix. It is interesting to note, however, that *S. theobromae* is placed as sister to *Archilejeunea* (Spruce) Schiffn., which it resembles in gynoecial features (Gradstein, 1994; Gradstein et al., 2001). More work with a more variable marker is clearly needed to determine the relationship of *S. theobromae*.

The systematic position of Myriocolea irrorata

In both MP and ML analyses *Myriocolea irrorata* is placed in the subfamily Lejeuneoideae as sister to *Cololejeunea*. Until recently, this species was only known from the original collection made by Richard Spruce in Ecuador in 1855. The lack of sufficient material has resulted in difficulties in morphological study and subsequent systematic placement. The rediscovery of this monospecific genus by Gradstein et al. (2004) at the original collection site allowed for molecular analysis and renewed morphological study. The species is robust and relatively large in comparison with other members of Lejeuneoideae. Apart from its size it is easily recognized by its large, undulate-crisped leaves without a trace of a lobule, and the numerous short branches, each forming a complex branch system terminating in heads of numerous clustered perianths (Thiers, 1983). Upon renewed morphological investigation, Gradstein et al. (2003, 2004) discovered the presence of pendular leaf segmentation and consequently suggested a close relationship to *Myriocoleopsis* Schiffn. and other members of the *Cololejeunea* clade. In our ML analysis *Myriocolea* and *Cololejeunea* are placed together as sister genera albeit with no support; in the MP tree *Myriocolea*, *Diplasiolejeunea*, and *Cololejeunea* form a moderately supported polytomous clade.

The relationships between Lejeunea, Macrolejeunea, *and* Microlejeunea

Both trees show a well-supported *Lejeunea* clade comprising several *Lejeunea* species and *Macrolejeunea pallescens* (Mitt.) Steph. *Microlejeunea ulicina* (Taylor) A. Evans is placed as sister to *Lejeunea* (including *Macrolejeunea*) with significant support. When the genus *Lejeunea* was first erected, authors adopted a broad concept. Over the years many species have been subsequently removed from the genus resulting in a deficit of characters left to define *Lejeunea* and related groups (Reiner-Drehwald, 1999). Although it is widely accepted that *Lejeunea* and several other genera such as *Mi-*

crolejeunea and *Macrolejeunea* are closely related, the systematic relationships of these taxa are still controversial. Although earlier authors considered *Macrolejeunea* to be a separate genus to *Lejeunea,* it is now often recognized as a subgenus of *Lejeunea* due to minimal morphological differences (Grolle, 1988). Our results suggest that *Lejeunea* s. str. is not monophyletic and hence would even support a synonym status of *Macrolejeunea,* which is nested in *Lejeunea.* A more definite statement should await further work with a more variable marker.

Some authors have regarded *Microlejeunea* as a subgenus of *Lejeunea* (e.g., Schuster, 1980), whereas others treat it as a separate genus (e.g., Reiner-Drehwald, 1994; Grolle, 1995, Gradstein & Costa, 2003). Apart from being smaller than most members of the genus *Lejeunea,* the zigzag stems with remote, hardly spreading leaves that possess very large lobules and the presence of ocelli in leaf lobes set it apart from species of *Lejeunea.* The position of *Microlejeunea* at the base of the *Lejeunea* clade can clearly be interpreted as both that of a subgenus of *Lejeunea* or a separate genus. A greater taxon sampling within the *Lejeunea* clade is needed to further clarify the systematic position of *Microlejeunea.*

The subdivision of Ceratolejeunea

Our results show a robust *Ceratolejeunea* clade with two distinct groups. Based on morphology, Dauphin (2003) split the genus into two subgenera, *Ceratolejeunea* and *Caduciloba* R. M. Schust. The smaller subgenus, *Ceratolejeunea* containing the type *C. grandiloba,* is characterized by entire underleaves, bulbous perianth horns, and the absence of reduced lobules. The larger subgenus, *Caduciloba* containing 19 species, is characterized by bifid underleaves, non-bulbous perianth horns, and the presence of reduced lobules. The recognition of two subgenera is supported by our analyses, with the type *C. grandiloba* shown as a well-supported sister group to the two representative species of the subgenus *Caduciloba, C. cornuta,* and *C. filaria.*

The Omphalanthus *clade*

A well-supported clade including species of *Omphalanthus, Cheilolejeunea,* and *Aureolejeunea* is detected in both the ML and MP trees. This clade is morphologically characterized by the presence of a distal hyaline papilla on the lobule, leaf cells with a few, very large oil bodies, the frequent possession of undivided underleaves, and numerous rosettes on spore surfaces (Gradstein et al., 2003). This clade has been discussed by several other authors (e.g., Gradstein et al., 1981; Weis, 2001) and corresponds to the tribe Cheilolejeuneeae R. M. Schust. of Schuster (2001). *Cheilolejeunea lineata* was also recovered as a member of this clade in both analyses. This result is noteworthy as this species has long been placed in the monospecific genus *Cystolejeunea,* separated from *Cheilolejeunea* by its strongly swollen lobules and ± flattened perianth without innovations and a very short beak (Evans, 1906; Gradstein et al., 2001). Moreover, the hyaline papilla on the leaf lobule was questionably considered to be proximal in this genus, whereas in *Cheilolejeunea* it is always distal in position. However, our study of material of *C. lineata* collected by A. Schäfer-Verwimp (Guadeloupe, *Schäfer-Verwimp 22183,* GOET) revealed that the lobule within the species may vary in size and is sometimes small as in *Cheilolejeunea.* Furthermore, we detected the hyaline papilla to be distal in position, not proximal. Oil bodies in *C. lineata* are also very large, as in *Cheilolejeunea.* Since the absence of innovations is not uncommon in *Cheilolejeunea* (e.g., in *C. adnata* (Nees) Grolle, *C. exinnovata* E. W. Jones, *C. obtusilobula* Horik.), this character is also not suitable to separate *Cystolejeunea lineata* from *Cheilolejeunea.* Following the morphological and molecular results, the monospecific genus *Cystolejeunea* is reduced to synonymy under *Cheilolejeunea*:

Cheilolejeunea (Spruce) Schiffn., in Engler & Prantl, Nat. Pflanzenfam. 1 (3): 124. 1893. Type species: *Cheilolejeunea aneogyna* (Spruce) A. Evans.

Cystolejeunea A. Evans, Bull. Torrey Bot. Club 33: 16. 1906, syn. nov. Type species: *Cystolejeunea lineata* (Lehm. & Lindenb.) A. Evans (*Cheilolejeunea lineata* (Lehm. & Lindenb.) Steph.).

2. THE *NAD*4–2 SPACER REGION MAXIMUM LIKELIHOOD TREE

The slow-evolving mitochondrial DNAs have previously been utilized to infer the phylogeny of bryophytes above family level (e.g., Nickrent, 2000; Beckert et al., 2001; Pruchner et al., 2002). In our preliminary analysis of the *nad*4–2 spacer region (Fig. 3), Lejeuneaceae and Jubulaceae form separate clades in agreement with the *rbc*L topology. The *nad*4–2 spacer region is very conserved, with only nine variable positions over the entire 183 base pairs used in the analysis (Fig. 3), but the results shown here warrant further investigation into its suitability as a marker for Lejeuneaceae.

Being of 26 bp long with only one variable position, the spacer region itself, however, is deemed unsuitable for phylogenetic analysis of larger taxon samplings.

General Conclusions

The systematic study of bryophytes has traditionally been conducted by analysis of morphological characters; however, due to the scarcity of characters for many genera, this has often led to conflicting hypotheses. Recent work with molecular data has been of paramount importance in increasing our understanding of this basal lineage of land plants. Extensive studies have been carried out on the mosses (e.g., Buck et al., 2000; La Farge et al., 2002; Tsubota et al., 2002; Pedersen et al., 2003) and thallose liverworts (e.g., Long et al., 2000; Wheeler, 2000; Boisselier-Dubayle et al., 2002), but little work has as yet been done on the leafy liverworts (e.g., Heinrichs, 2002; Ahonen et al., 2003; Groth et al., 2003). Lejeuneaceae are the largest family of liverworts, and although extremely rich in morphological variation, the systematic placement of several genera and species remains unresolved.

The chloroplast gene *rbc*L appears to be relatively conserved within Lejeuneaceae, but despite this our preliminary results go some way to explaining the systematic placements of several genera within the family. Within the subfamily Lejeuneoideae, *rbc*L is especially useful for resolving relationships at genus and even species level. Other major molecular studies on large bryophyte families have often led to results that were non-congruent with previous morphological hypotheses (e.g., La Farge et al., 2002; Werner et al., 2002; Vanderpoorten et al., 2002, 2003). In contrast, results seen to date in Lejeuneaceae can generally be explained by morphological evidence. The existence of a third smaller clade in our results containing *Schiffneriolejeunea* and *Symbiezidium*, however, is not supported by morphology. It must be said that these results are initial ones and with further markers and greater taxon sampling, positions of several genera could change. We intend to work with a more variable marker such as nr ITS to help resolve the relationships within Lejeuneaceae further. Groth-Malonek et al. (2004) demonstrated that the ITS region is a valuable marker to resolve the relationships within closely related genera of Lejeuneaceae but not at deeper nodes. Ahonen et al. (2003) also illustrated the usefulness of parts of the ITS region in resolving relationships between members of Lejeuneacae. We are convinced that combined analysis of *rbc*L with a more variable marker such as ITS or *trn*L-*trn*F will produce a well-resolved topology within Lejeuneaceae.

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SYSTEMATICS OF THE BRYOPHYTA (MOSSES): FROM MOLECULES TO A REVISED CLASSIFICATION[1]

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Abstract

Two hundred years of bryological investigations of morphological, anatomical, and developmental characters form the foundation for systematics concepts in mosses, and hence for their classification. With the development of phylogenetic theory and more recently of techniques allowing the extraction of DNA and the amplification and sequencing of specific loci, a new source of characters has become available to test systematic hypotheses. In the last decade over 100 phylogenetic studies of mosses have been published. These have led to the revision of many supraspecific taxonomic circumscriptions. Indeed, many taxa, whether these are genera, families, or orders, have been shown to be paraphyletic or polyphyletic. The revised lineages may satisfy a criterion of monophyly, but in some cases they can no longer be diagnosed using traditional morphological characters. Although phylogenetic inferences are shaping our systematic concepts, the significance of the contributions remains to be tested by future studies. Indeed, several new hypotheses are only weakly supported. Only recently have inferences been made from multiple loci spanning all three genomic compartments, and structural constraints on the evolution of molecules are only beginning to be integrated in analytical assumptions. Here we review most studies that have addressed systematic hypotheses and, based on these results, have amended our recent classification of mosses. The following new taxa are proposed: Takakiopsida (Crand.-Stotl.) comb. et stat. nov., Andreaeaobryopsida (B. M. Murray) comb. et stat. nov., Oedipodiopsida (Schimp.) comb. et stat. nov., Oedipodiales (Schimp.) comb. et stat. nov., Tetraphidopsida (M. Fleisch.) comb. et stat. nov., Scouleriales (S. P. Churchill) comb. et stat. nov., Bryanae (Engl.) comb. et stat. nov., Rhizogonianae (M. Fleisch.) comb. et stat. nov., Rhizogoniales (M. Fleisch.) comb. et stat. nov., and Pylaisiadelphaceae fam. nov.

Key words: Bryophyta, classification, mosses, phylogeny.

The systematics of bryophytes s.l. has undergone a renaissance following the adoption of techniques extracting the phylogenetic signal carried by molecular characters. DNA extraction, RFLP analysis, and PCR-based amplification made their entry into bryological laboratories slowly, and only after the techniques had become routinely applied in mycological and vascular plant laboratories. The slow start may be explained by the difficulty in growing bryophytes in culture and the need, at least in early extraction protocols, for large amounts of material (e.g., one gram), a requirement not easily satisfied by most bryophytes. Now small quantities in the order of a few mg (e.g., a single operculate capsule, or even a section of fresh tissue) may suffice to yield enough DNA to serve as a template for a successful amplification. Although the first phylogenetic study including sequences of bryophytes was published in the mid 1980s (Hori et al., 1985), it took another seven years before the next such research projects were published (Mishler et al., 1992; Waters et al., 1992). Another period of latency followed, until the field of molecular phylogenetic systematics attracted the interest of various bryological laboratories (see Goffinet & Hax, 2001, and Goffinet, 2003, for bibliographies).

Following the annual meeting of the American Bryological and Lichenological Society in Montreal in 1997, research groups coordinated their efforts in addressing the major questions pertaining to the phylogeny of mosses, with the objective of providing a first summary of the potential and the contributions of sequence data to moss systematics at the International Botanical Congress in St. Louis in 1999. The initiative was sponsored by the Green Plant Research Coordination Group led in part by Brent Mishler (University of California, Berkeley). The proceedings of the symposium were published in *The Bryologist* the following year (Goffinet & Hedderson, 2000). All hypotheses that were tested pertained to ordinal or familial relationships. Indeed, few of these studies sampled DNA characters

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to test phylogenetic hypotheses at the species (e.g., Shaw & Allen, 2000) or population level (Shaw, 2000a).

In the last three years researchers have broadened the scope of their studies, although the focus remains on elucidating the relationships among major lineages of bryophytes. Here we provide a review of the progress made in moss molecular systematics since the last botanical congress, the problems that have surfaced, and the perspectives that the field offers the bryological community. Although molecular markers have been critically explored for their signal to resolve the relationships among lineages of extant land plants, and in particular to test the monophyly of the Bryophyta (i.e., mosses) sensu Buck and Goffinet (2000), reviews of contributions to this field of study are available elsewhere and will not be repeated here (cf. Duff & Nickrent, 1999; Goffinet, 2000).

Molecules and Systematics

The objectives of systematics are to elucidate the relationships among taxa. Taxonomy deals with the recognition of taxa, with the main focus being on species. Two hundred years of bryological studies have led to the recognition of about 12,000 species of mosses (Crosby et al., 2000). Morphological characters have been central to our systematic hypotheses and have been the basis for the most widely adopted species concepts. These characters are the phenotypic expression of a genotype, and the phenotype may vary along an ecological gradient. Furthermore, most characters are continuous in nature rather than binary. Depending on the sample size, and one's knowledge of the group under consideration, the variation may be viewed as patterned or continuous and can determine the taxonomic concepts and consequently our phylogenetic predictions.

Genetic changes serve as another source of characters that can be used to test our hypotheses. The question is not whether they are better, more reliable, and hence more accurate than morphological characters. Indeed, if species are real and the product of evolution, there should be defined fixed changes in their genomes. Hence, the null hypothesis is that if our morphology-based hypotheses are correct, inferences from variation in molecular makers should strengthen them. The outcome of any test can be congruence or incongruence. The debate about the usefulness of molecular markers is fueled by cases of incongruence between the two sources of characters. Although the trend may be for molecular systematists to preferentially adopt the phylogenetic or genealogical pattern as the truth, and hence assume that inferences from morphological characters are misled by incorrect assumptions of homology (i.e., homoplasy), incongruence should, in fact, be the starting point for future research to elucidate the underlying nature of this incongruence. Few systematists engage in testing the stability of the traits under artificial conditions or their homology assumptions of morphological characters (typically based on levels of similarities of the end product of development) by seeking support from the ontogeny of these traits. Similarly, the constraints that shape the evolution of specific loci are often neglected, the orthology (and hence homology) of the sequences is often merely assumed, and the alignment itself may not be dictated by structural features of the gene product. Hence, incongruence can be accounted for by several sources of error. However, if we assume that all these a priori tests for homology were performed, inferences from morphological and molecular data sets may still lead to incongruence. The most likely sources for this may be caused by convergent evolution evident in morphological characters or lateral transfer of genetic material. Morphological reduction may lead to the loss of complex traits that are diagnostic (i.e., synapomorphies) of a lineage. Consequently, the reduced taxa display the symplesiomorphic state. Depending on the extent of the reduction, long branches may characterize these taxa in the true phylogenetic tree, and be pulled to the base in the reconstructed phylogeny. Morphological reduction is likely not paralleled by reversals to ancestral traits in DNA sequences, and hence it is in these cases that lay the potential and the contribution of molecular markers. Reduction may indeed be a common phenomenon in the evolution of bryophytes (Hyvönen et al., 2004). Character loss and neoteny are not the only sources of convergence. Parallel forward evolution (i.e., not reversals) characterize the independent acquisition of phyllodioicy in three subfamilies of the Brachytheciaceae (Huttunen & Ignatov, 2004), and within the Hookeriales a double costa has arisen at least six times and a laminal limbidium at least five times (Buck et al., 2004).

Lateral transfer of genetic material (i.e., hybridization) had been discounted as an evolutionary process shaping bryophyte diversification (Vitt, 1971). Several studies, in particular in *Sphagnum* (Såstad et al., 1999, 2000; Shaw & Goffinet, 2000), the Mniaceae (Wyatt et al., 1988), and the Polytrichaceae (Derda & Wyatt, 2000), have demonstrated the existence of several taxa of hybrid origin. Since hybrids retain discrete portions of their genome

from each parent, topological incongruence is not restricted to morphological versus molecular data, but also to loci from distinct genomic compartments, or maybe even between nuclear loci, if recombination took place following hybridization. The significance of hybridization as a process in bryophyte evolution is not clear, but evidence pointing to its widespread occurrence is growing (e.g., van der Velde & Bijlsma, 2004).

Molecular characters are thought to test homology assumptions made from morphological character states across systematic ranks and evolutionary lineages and, thereby, test morphology-based phylogenetic hypotheses. Here we review the studies aimed at testing the circumscription and relationships of supraspecific taxa of mosses, with a focus on families and orders.

Phylogenetic Reconstructions Among Mosses

early radiation of mosses

The early diversification of mosses is correlated with conspicuous transformations in the mode of sporangial dehiscence and, even more so, in spore dispersal mechanisms (Schofield, 1985). In most mosses the sporangium opens with the loss of an apical operculum, which forms a lid on the capsule. In *Takakia*, dehiscence follows a single spiral longitudinal line, and in the Andreaeopsida and Andreaeobryopsida, four (or sometimes more) straight lines stretch between the poles of the capsules. Among operculate mosses, *Sphagnum* lacks a peristome, whereas the vast majority of remaining mosses have teeth lining the mouth of the sporangium. The lack of a peristome among these derived mosses is considered the result of a reduction (i.e., loss) except maybe for *Oedipodium* (see below). Peristomate mosses and *Oedipodium* share a cylindric columella compared to the dome-shaped columella present in *Takakia*, the Sphagnales, and the Andreaeales, as well as the presence of protonematal side-branch initials (Newton et al., 2000).

Mishler and Churchill (1984) proposed the first formal phylogenetic hypothesis for the mosses. Their sampling did not include *Takakia*, whose affinities to the mosses were still unsubstantiated at the time, or *Andreaeobryum*, which had, however, already been described (Steere & Murray, 1976). Mishler and Churchill (1984) rooted their tree, in the absence of an explicit outgroup, with *Sphagnum*. Following their phylogeny, the following lineages arose sequentially through time: Andreaeales, Tetraphidales, Polytrichales, Buxbaumiales, and ultimately the Bryales. This hypothesis remained unchallenged for over a decade.

Based on variation in 18S (nrDNA), Hedderson et al. (1996, 1998) examined the relationships among the three main lineages of the Bryophyta s.l. Their sampling included nine mosses. They provided the first molecular evidence in support of *Takakia* belonging to the mosses, as suggested by gametangial and sporophytic characters (Smith & Davidson, 1993). Although the mosses were well supported as a monophyletic group, the relationships within this lineage lacked Bremer support. The hypothesis that *Takakia*, *Andreaea*, and the nematodontous mosses shared a unique common ancestor, and were sister to the arthrodontous mosses, remained tentative at best. Based on the mitochondrial locus *nad*5, Beckert et al. (1999) resolved *Sphagnum* as the most basal bryophyte. *Takakia* was missing from their taxon sampling. The peristomate mosses appeared as polyphyletic, as the Andreaeales were nested between the Polytrichales and the remainder of the peristome-forming mosses. However, the relationships among these major lineages also lacked support. Although Hyvönen et al. (1998) focused on the Polytrichales, they were the first to highlight the phylogenetic significance of *Oedipodium*. Rather than being allied to the Splachnaceae in the arthrodontous mosses as suggested by Vitt (1984), *Oedipodium* was resolved as sister to the Polytrichaceae (see also Hyvönen et al., 2004). This hypothesis has now been corroborated by other studies (Newton et al., 2000; Goffinet et al., 2001; Cox et al., 2004). Whether the absence of a peristome in *Oedipodium* is a true plesiomorphy or results from secondary loss remains unclear (Cox et al., 2004). The sister-group to *Oedipodium* cannot be defined by a single character except the presence of a peristome (Newton et al., 2000)

The first phylogenetic reconstruction to include an exhaustive sampling of ordinal exemplars (orders sensu Vitt, 1984) as well as *Takakia* and *Andreaeobryum* was presented by Newton et al. (2000). Their inferences from data combining sequences of the nuclear and chloroplast genomes and morphology led to an evolutionary scenario wherein *Takakia* and *Sphagnum* form a weakly supported basal lineage, followed by the Andreaeales (including *Andreaeobryum*), and finally those mosses with a cylindrical columella. The position of the Andreaeales between the *Takakia-Sphagnum* clade and the remaining mosses was well supported, but monophyly of the order received, by contrast, only weak support from the data. These hypotheses have most recently been tested by Cox et al. (2004), who inferred the succession of cladogenic events from variation in eight loci from all three genomes, using a set of representative taxa for each major lineage

of mosses sensu Vitt (1984). Their results concur with those of Newton et al. (2000), and, in fact, none of the ambiguities pertaining to the early diversification has been clarified despite this intensive character sampling and the application of distinct models of evolution to discrete partitions of the sequences. The failure to resolve the order of early cladogenic events is not inherent to the genetic data, as inferences from morphology alone also resulted in ambiguous affinities for *Takakia, Andreaea,* and *Andreaeobryum* (Newton et al., 2000). The reconstruction of ancient evolutionary events is problematic not only for mosses, but is a problem that plagues systematists working on flowering plants, invertebrates, and fungi. Much hope is being placed on the exploration of structural features of complete genomes to resolve these impasses (see, for example, Kelch et al., 2004).

EARLY RADIATION OF THE PERISTOMATE MOSSES

As mentioned above, one clear contribution of molecular data to our understanding of moss evolution has been the identification of *Oedipodium* as the sister-group to the peristomate mosses. Based on the primary architecture of the teeth, peristomes are either nematodontous (i.e., composed of whole cells) or arthrodontous (i.e., composed mainly of periclinal walls belonging to the cells of two or three [rarely more] concentric layers of cells of the amphithecium; Vitt, 1984; Schofield, 1985). The Tetraphidales and Polytrichales both have nematodontous peristomes. The two orders differ in the number and size of the teeth (four vs. 16, 32, 64, or more, respectively) and the ontogeny of the peristome. Based on the study by Wenderoth (1931), the development of the peristome of the Polytrichales is considered to be characterized by an early additional anticlinal division, which leads to a doubling of the number of cells in all amphithecial layers compared to other mosses. Furthermore, the divisions in the inner peristomial layer (IPL) all appear to be symmetric in *Polytrichum,* whereas in *Tetraphis* the critical division that leads to 16 cells in the IPL appears, albeit slightly, to be asymmetric (Goffinet et al., 1999; but see Shaw & Anderson, 1988, who argue for a symmetric division). Another sporangial character that distinguishes the two is the lack of air spaces in the Tetraphidales. This character state is shared with other basal lineages (*Takakia, Sphagnum,* and the Andreaeales; Goffinet et al., 2001).

The hypothesis that the nematodontous peristome defines a monophyletic lineage has never been explicitly tested, but instead has always been discussed in the context of moss evolution, and hence has never received the attention of taxon and character sampling that this important question deserves. Mishler and Churchill (1984) proposed that the nematodontous mosses were paraphyletic on the basis of the air space character mentioned above. Vitt (1984: 742–743) considered the peristome of the Polytrichales and Tetraphidales to be developmentally unrelated, and even raised the possibility that the Tetraphidaceae belong to the Bryales.

Inferences from *nad*2 and *nad*5 sequences suggest that the nematodontous mosses are monophyletic (Beckert et al., 2001). Paraphyly is supported by characters from the two other genomes (e.g., Hyvönen et al., 1998; Newton et al., 2000; Magombo, 2003a), although preliminary inferences by Hedderson et al. (in Vitt et al., 1998) from 18S sequences favored the common ancestry for the group. Support for monophyly of the nematodontous mosses (Beckert et al., 2001) seems stronger than that gathered in favor of paraphyly (e.g., Newton et al., 2000). The strength of the support may depend on the parameter chosen to measure it. Cox et al. (2004) showed that the clade comprising the Tetraphidales and the arthrodontous mosses received high posterior probabilities but low bootstrap percentages. This may be congruent with the relative short branches that unite these taxa. Within a paraphyletic scenario the order of evolution also remains ambiguous. Did the evolution of the Polytrichales precede that of the Tetraphidales or vice versa? Although most optimal reconstructions point to the former (except Magombo, 2003a), all the branches of interest are short and support values are virtually lacking. So far, taxon sampling within the Tetraphidales, which includes but five species (Crosby et al., 2000), has been limited to *Tetraphis pellucida* Hedw., and hence inferences are not considering the potential significance of *Tetrodontium.* The resolution of the relationships between the Tetraphidales and Polytrichales is essential for assessing the significance of the nematodontous peristome in the evolution of the arthrodontous teeth. Central to this question is also the position of the Buxbaumiales.

ORIGIN OF THE ARTHRODONTOUS MOSSES

The Buxbaumiaceae and the Diphysciaceae may be considered distinct families (e.g., Brotherus, 1925) or not (e.g., Vitt, 1982, 1984), but in either case they have traditionally been accommodated within a single lineage (e.g., Buxbaumiales by Brotherus, 1925, and Vitt, 1982, or Buxbaumiineae by Vitt, 1984). Although *Buxbaumia* and *Diphys-*

cium differ in a number of characters (see Magombo, 2003a), they share a unique peristomial architecture (Edwards, 1984). The inner peristome consists of a high, pleated membrane. In *Buxbaumia* the number of cells in each amphithecial layer is double that found in *Diphyscium* and most mosses (and is similar to the pattern found in *Polytrichum* (Wenderoth, 1931)), and hence *Buxbaumia* has 32 rather than 16 pleats. In *Diphyscium* the outer peristome is composed of a single row of teeth that is fused to the inner membrane. In *Buxbaumia* the cell walls of one or two outer peristomial layers of cells may be thickened and partially resorbed, and thus may form two rows of teeth, even if the outermost row is rudimentary and remains partially attached to the capsule wall (Edwards, 1984). Finally, in *Buxbaumia* the cells surrounding the outer peristome are entire and extremely thickened. Whether this peristome is homologous to the nematodontous peristome has been much discussed by early workers (see review in Edwards, 1984), and understandably so. Homology of these structures would suggest that the peristome of the Buxbaumiaceae is intermediate between the arthrodontous and nematodontous architectures and hence may mark the transition from one to the other. If the peristome of the Buxbaumiaceae shares anatomical features with the nematodontous teeth due to convergence then the affinities of the Buxbaumiaceae may not lay near the Polytrichales. Vitt (1982), for example, considered the Buxbaumiales to be arthrodontous and later even suggested that this lineage was ancestral to the haplolepideous mosses (Vitt, 1984). Most other workers treated the Buxbaumiales (as orders or suborders) as distinct from the arthrodontous mosses, with close ties to the Polytrichaceae (e.g., Brotherus, 1925) or in a truly intermediate position (e.g., Crosby, 1980; Mishler & Churchill, 1984).

Shaw et al. (1987) demonstrated that the inner peristome in *Diphyscium* is homologous in position to the endostome of arthrodontous mosses and that the development of this membrane follows a pattern identical to that observed in haplolepideous mosses. In fact, these authors concluded that based on morphological and ontogenetic data, *D. foliosum* is fundamentally haplolepideous.

Clearly, ambiguous homologies and overall phenetic isolation have fueled much of the controversy regarding the systematic position of the Buxbaumiales and their phylogenetic significance over the last century and more. Inferences from mitochondrial loci (Beckert et al., 1999, 2001), chloroplast loci (Goffinet et al., 2001), or combined nuclear and cytoplasmic loci (Newton et al., 2000; Cox et al., 2004) suggest that *Diphyscium* is the closest extant relative to the arthrodontous mosses. Hence, the Buxbaumiaceae sensu Vitt (1984) are consistently resolved as a paraphyletic entity, with *Buxbaumia* having evolved prior to the differentiation of arthrodontous mosses from *Diphyscium* (Magombo, 2003a). Goffinet et al. (2001) suggested, on the basis of inferences from the plastid gene *rps*4, that *Buxbaumia* may be closely related to the Tetraphidaceae, but this hypothesis has subsequently been refuted. *Buxbaumia*, *Diphyscium*, and the arthrodontous mosses share a significant deletion in the *rps*4-*trn*S_{UGU} intergenic spacer (Cox et al., 2004).

The resolution of the sister-group to the arthrodontous mosses could have significant implications regarding the evolution of peristome types in mosses. However, given the paraphyly of the Buxbaumiaceae sensu Vitt (1984), *Diphyscium* can no longer serve as an exemplar for the family. Furthermore, it is impossible on the basis of the phylogeny alone to argue for or against the homology of the peristome and the nematodontous architecture. It is imperative that the ontogeny of the peristome of *Buxbaumia* be described to complete the survey of peristome development.

EARLY DIVERSIFICATION OF THE ARTHRODONTOUS MOSSES

Shaw et al. (1987) argued that the development of the peristome of *Diphyscium* follows a pattern consistent with that of haplolepideous mosses: the division in the inner peristomial layer (IPL) that results in 16 cells is clearly asymmetric, and this division is typically followed by one or, rarely, two more cytokinetic events, resulting in typically 24 cells in the IPL. The peristomial formula (after Edwards, 1984) is 4:2:3, and prior to cell-wall thickening and resorption, the amphithecium of *Diphyscium* and that of the haplolepideous mosses is identical. We now know that this similarity is not indicative of close affinities between these taxa but should instead be viewed as evidence that the 4:2:3 pattern and the asymmetry of the IPL division is plesiomorphic in mosses. If so, is the haplolepideous condition basal among the arthrodontous mosses?

The Bryopsida sensu Buck and Goffinet (2000) accommodate nearly 12,000 species, which can be arranged into three groups based on their peristomial architecture (Philibert, 1884–1890, and translated by Taylor, 1962). The haplolepideous peristome or *Dicranum*-type peristome is defined by the endostome segments being composed of a single column of cell plates on their outer surface. The

division in the IPL that leads to the 16-cell stage is asymmetric (Shaw et al., 1989b). The exostome is highly reduced and most often completely lacking. By contrast, the exostome is characteristic of the diplolepideous mosses, but the term "diplolepideous" refers not to the two rings of teeth, but to the two columns of dorsal plates on the outer teeth. Two main types of diplolepideous peristomes can be recognized, and these are typified by the peristomes of *Funaria* and *Bryum*. In the former, the endostome segments are positioned opposite the exostome teeth (diplolepideous opposite peristome), whereas in the latter, the teeth and segments alternate (diplolepideous alternate peristome). Furthermore, the IPL undergoes a symmetric division in *Funaria* (Shaw et al., 1989a; Schwartz, 1994), whereas in *Bryum* that division is clearly asymmetric (Shaw et al., 1989a). The *Bryum*-type peristome differs further by the presence of cilia between the segments and hence positioned opposite the outer teeth (Vitt, 1981).

The relationships among the haplolepideous, diplolepideous alternate, and diplolepideous opposite mosses have been inferred implicitly on the basis of these peristomial features (e.g., Vitt, 1981, 1984). However, as pointed out by Vitt et al. (1998), these characters alone fail to yield a robust hypothesis because (1) there are too few characters, (2) lineages are defined by character combinations and not individual characters, and, hence, (3) individual characters are incongruent among themselves. Thus, it is not surprising that the relationships within the Bryopsida remained unresolved.

Most inferences from cytoplasmic genome-encoded protein loci suggest that the diplolepideous mosses are paraphyletic, with the haplolepideous mosses forming a sister-group to those species with a *Funaria*-type peristomial architecture and the Encalyptaceae (hypothesis I; e.g., Beckert et al., 1999, 2001; Goffinet & Cox, 2000; Goffinet et al., 2001). Under this scenario, the haplolepideous mosses and the *Funaria*-type peristomate mosses would share an opposite arrangement of their peristomes (Vitt et al., 1998; Goffinet et al., 2001). By contrast, inferences from nuclear-encoded genes (Hedderson et al., 1996; Capesius & Stech, 1997) resolve the haplolepideous mosses as sharing a common ancestor with the diplolepideous alternate mosses (i.e., those with a *Bryum*-type peristome; hypothesis II); here the asymmetric division in the IPL serves as a synapomorphy linking the haplolepideous mosses to a group of mosses with diplolepideous peristomes. When the data are combined the overall signal is ambiguous and may favor the first (Goffinet & Cox, 2000) or the second hypothesis (Cox et al., 2000, 2004; Newton et al., 2000).

Paralleling the ambiguity of the relationships among these main lineages of the Bryopsida is the uncertainty regarding the affinities of *Timmia*. The peristome of *Timmia* is double, and its architecture is clearly that of a diplolepideous moss as the dorsal surface of each exostome tooth is derived from two columns of cells (Murphy, 1988). However, its endostome differs from the typical *Funaria*-type or *Bryum*-type in that it is divided into 64 segments, which are reminiscent of cilia. Vitt (1984) and others must have considered this peristome to be derived from a *Bryum*-type situation because *Timmia* is historically included in the Bryales or Bryineae, depending on the classification scheme. The hypothesis that *Timmia* is a member of the Bryales sensu Vitt (1982) or Crosby (1980) is refuted by all phylogenetic analyses of DNA sequences. Hypotheses regarding its precise affinities are, however, incongruent among the various studies. *Timmia* has been reconstructed as basal among arthrodontous mosses (Goffinet & Cox, 2000 [their combined data set]; Tsubota et al., 2003 [*rbc*L]), as sister to the Dicranales-Funariales-Encalyptales clade or sister to only the Funariales based solely on cytoplasmic DNA (Beckert et al., 1999, 2001; Goffinet & Cox, 2000; Goffinet et al., 2001), or as marking the transition between the Funariales and the remaining arthrodontous mosses when the phylogenetic signal of genomic markers is combined (Cox et al., 2000; Newton et al., 2000).

Although it is evident that a formal reconstruction of peristome transformations should not be done in the absence of a robust phylogeny, it is not clear to what extent *Timmia* will be informative toward elucidating the evolution of peristome types. Indeed, the interpretation of evolutionary trends will be biased by the a priori homology assumptions that are made. Are the "cilia" of *Timmia* homologous to those of *Bryum*? Are the segments of *Funaria* and *Bryum* analogous and derived independently through fusion of the "cilia" of *Timmia*? Cox et al. (2004) considered the peristome of *Timmia* to be of the opposite diplolepideous type and concluded, given the basal position of the genus, that this peristome type was plesiotypic and hence ancestral to the diplolepideous alternate type. The underlying assumption of this hypothesis needs to be substantiated.

Molecular data may be essential for providing the underlying phylogenetic hypothesis, but developmental characters are imperative, although not sufficient, to clarify homology assumptions between the peristome of *Timmia* and other arthrodontous

mosses. The early radiation of arthrodontous mosses remains basically unresolved. The incongruence among studies may be caused by (1) inconsistencies in the taxon sampling, and hence be an artifact of the analyses, (2) lack of a strong phylogenetic signal in one or more data partitions, (3) inappropriate assumptions regarding the models of evolution of the partitions, or (4) reflect true incongruence between genomes due to lateral gene transfer. This final hypothesis (the fourth) seems likely considering the congruence among cytoplasmic genomes, and their incongruence with nuclear DNA. To test for ancient hybridization, inferences from nuclear, mitochondrial, chloroplast, and combined loci must be compared for identical sets of taxa. So far, such analyses are lacking.

SYSTEMATICS OF MAJOR LINEAGES OF MOSSES

Reconstruction of the backbone phylogeny of mosses has only consumed a portion of the efforts invested in inferring phylogenies from molecular data. Many studies focus on individual lineages and, increasingly, on the relationships within orders and families. Although the taxonomic spectrum covered continues to expand, notable gaps exists, such as the lack of investigation in the Andreaeales sensu Vitt (1984).

The following review aims at being comprehensive but will not include an in-depth discussion of the implications in terms of morphological evolution for all aspects of the phylogenies that have been proposed. Except for the Sphagnaceae and Polytrichaceae, generic or infrageneric relationships will not be addressed, unless genera are identified as polyphyletic based on DNA data.

The Sphagnaceae

The peatmosses comprise two genera, *Sphagnum* and *Ambuchanania*. The latter is monospecific and is based on *S. leucobryoides* Yamaguchi, Seppelt & Z. Iwats. (Yamaguchi et al., 1990), for which the original authors erected the section *Buchanania* within *Sphagnum*. This species is morphologically isolated within the genus, and Crum and Seppelt (1999) subsequently established a new genus, family, and even order within the Sphagnopsida (Crum, 2001). Whether the elevation of *S. leucobryoides* to the rank of genus or above results in *Sphagnum* being paraphyletic has been tested only once: Shaw (2000b) obtained partial sequences for the 26S gene (nrDNA) for *Ambuchanania*. His inferences resolve *Ambuchanania* as sister to the remainder of *Sphagnum* and thereby are congruent with the systematic concept of Crum and Seppelt (1999).

Given that some sequences (e.g., *psb*T and 26S) yielded insufficient variation to yield either resolution or support of the infrageneric phylogeny of *Sphagnum*, the sampling of molecular characters was extended and more taxa were included (Shaw, 2000b). In an unrooted tree four main clusters could be recognized. The cores of these groups correspond to the sections *Acutifolia*, *Cuspidata*, *Sphagnum*, and *Subsecunda* within *Sphagnum*. Smaller sections (e.g., sects. *Squarrosa* and *Rigida*) were synonymized, although their position in the combined tree did not violate the monophyly of larger sections (sects. *Acutifolia* and *Sphagnum*, respectively). Shaw et al. (2003a) subsequently focused on the backbone phylogeny of *Sphagnum* in order to polarize morphological character transformations, and to identify the sister group to section *Acutifolia*, which is the focus of their current research. Unfortunately, due to the scarcity and maybe the age of the original material (pre 1990), Shaw et al. (2003a) were not able to use *Ambuchanania* to root their reconstruction of *Sphagnum*. Their inferences were based on an unprecedented 16 loci composing a matrix with nearly 16,000 characters (yielding 712 parsimony informative characters) sampled for 24 species. Based on a preliminary analysis, constructed with approximately 10,000 characters and using *Takakia* and *Andreaea* as the outgroups, they identified *S. sericeum* Müll. Hal. and *S. lapazense* H. A. Crum as basal taxa in *Sphagnum*. In the rooted tree, these two species form a grade. They are consistently (i.e., for every locus) well differentiated from other Sphagna, and at least *S. sericeum* had been considered a potential candidate for an ancestral position in the genus. However, in his previous study Shaw (2000b) tentatively concluded that *S. sericeum* was a member of section *Acutifolia*. His caution, which proved justified (Shaw et al., 2003a), was based on the fact that only one locus (i.e., ITS) had been sequenced for this species and that hybridization, although not verified for this taxon, has been demonstrated as an evolutionary process within the genus (Shaw & Goffinet, 2000).

The relationships among the main sections of *Sphagnum* could not be resolved or well supported due to the paucity of characters in the data set (see fig. 1 in Shaw et al., 2003a). The inclusion of additional and more variable regions whose alignment is less unambiguous within the ingroup, and rooting the tree with *S. sericeum* and *S. lapazense*, the remainder of the Sphagna compose two major lineages that were well supported by posterior probabilities but lack bootstrap support (see fig. 2 in Shaw et al., 2003a). The first lineage comprises the

sections *Sphagnum*, *Rigida*, and *Cuspidata*. Their monophyly is well supported but their relationships are not. Similarly the relationships among three sections in *Sphagnum* (i.e., *Acutifolia*, *Squarrosa*, and *Subsecunda*) composing the second lineage are ambiguous, and the affinities of *S. wulfianum* Girg. (sect. *Polyclada*) within this clade are unresolved. Whether the basal branches can be characterized by transformations in morphological characters, and hence whether the suprasectional clades can be defined by synapomorphies, remains to be addressed. The various studies by Shaw and colleagues have laid the foundation for future in-depth work on discrete sections of *Sphagnum*, and also have set what may soon or should become a standard in character sampling.

The Polytrichaceae

The Polytrichaceae share "a well developed conducting system, differentiated above-ground and below-ground portions and a complex leaf structure" (Vitt, 1982: 308), and to these could be added thigmotropism (Newton et al., 2000). All these characters, however, may not represent synapomorphies.

The complex leaf structure mentioned by Vitt (1982) likely refers to the presence of photosynthetic lamellae on the adaxial surface of the leaves. The sole comprehensive phylogenetic reconstruction of the family by Hyvönen et al. (1998) suggests that these structures evolved after the early diversification of the group, and hence serves to unite a clade within the family rather than characterize the family as a whole. Hyvönen et al. (1998) based their inferences on variation in a nuclear locus (18S), a chloroplast gene (*rbc*L), and morphological characters. Support for the monophyly of the family is drawn primarily from *rbc*L data. The 18S data set comprises few parsimony informative sites, and the morphological characters appear highly homoplastic and their analysis yields no resolution from other peristomate mosses used as outgroups. Similarly, support is lacking for relationships within the ingroup, except after successively weighting transformations, and then only for generic monophyly or a sister-group association between *Bartramiopsis* and *Lyellia*. Hence, no informative evolutionary interpretation could be made from their analyses. Broadening the character sampling to the *trn*L-*trn*F region (cpDNA) and partial sequences of the *nad*5 locus (mtDNA) and including more taxa led the total evidence analyses to converge on two most parsimonious trees (Hyvönen et al., 2004). Although the hypotheses of monophyly must be rejected for several genera (i.e., *Oligotrichum*, *Polytrichastrum*, *Pogonatum*, and *Polytrichum*), based on the criterion of maximum parsimony, the incongruence among characters seems significant considering the overall low bootstrap values characterizing most nodes. Moderate support characterizes the branch that separates the core of the Polytrichales from a basal grade composed of *Alophozia*, *Atrichopsis*, *Bartramiopsis*, and *Lyellia*. *Alophozia* and *Atrichopsis* compose a lineage sister to the remainder of the order. They lack the ventral lamellae that are typically seen as diagnostic of the Polytrichales. Thus, it is hypothesized that these structures were acquired after the early diversification of the Polytrichales, and hence cannot serve as a synapmorphy for the lineage.

The Diphysciaceae/Buxbaumiaceae

The Buxbaumiaceae as defined by Vitt (1984) are clearly paraphyletic, and *Diphyscium* appears as the sister-group to the arthrodontous mosses (Magombo, 2003a). The Buxbaumiaceae s. str. are monogeneric, whereas the Diphysciaceae traditionally included three genera. *Muscoflorschuetzia* and *Theriotia*, however, are resolved in a nested position within *Diphyscium*. The former is gymnostomous, and multistratose leaves distinguished the latter. Both these conditions must be interpreted as of late derivation. Hence, recognition of these genera as distinct taxonomic entities should be abandoned as proposed by Magombo (2003b; cf. Appendix 1 herein).

Phylogeny of the Funariales

Within the Funariales, Vitt (1982; or suborder in Vitt, 1984) included the Funariaceae, Disceliaceae, Pseudoditrichaceae, Gigaspermaceae, and Ephemeraceae. A single study (Goffinet & Cox, 2000) focused on the circumscription and phylogeny of this lineage. The affinities of the Pseudoditrichaceae have not yet been tested using DNA sequences, but Shaw (1984) interpreted the peristome as of the *Bryum*-type and hence transferred the family to the Bryales. *Goniomitrium* and *Ephemerum* have been transferred to the Pottiales by Goffinet and Cox (2000), and should be considered members of the Pottiaceae (cf. Appendix 1 herein). Neither of these taxa shares a deletion of one codon in the *rps*4 gene that is characteristic of the Funariaceae (Goffinet & Cox, 2000). The Funariales are best defined by the architecture of the leaf, and although *Discelium*, the Gigaspermaceae, and several Funariaceae are gymnostomous, all these taxa may share with peristomate Funariaceae a pattern of cell division in

the amphithecial layer wherein the divisions that lead to a 16-celled IPL are symmetric (Rushing & Snider, 1980; Shaw & Allen, 1985; Shaw et al., 1989a; Schwartz, 1994; Goffinet et al., 1999).

The Funariales may be most closely related to the Encalyptales. Morphological support for a shared ancestry is lacking given the highly divergent gametophytic and sporophytic architecture (Vitt, 1982, 1984), but may be found in the development of the amphithecium. Goffinet et al. (1999) considered the division in the IPL in *Encalypta* to be symmetric as in the Funariales, but ontogenetic evidence for this hypothesis has not been published.

The Catascopiaceae, a monospecific family traditionally placed in the Bryales (Vitt, 1984), was resolved within the Funariales-haplolepideous clade based on *rps*4 data (Goffinet et al., 2001). The diplolepideous peristome of *Catoscopium* is composed of an endostome that is reduced to a low hyaline membrane. The architecture of the peristome is, therefore, not informative to assess the ordinal affinities of *Catoscopium.* Virtanen (2003) further demonstrated that *Catoscopium* did not belong to the Bartramiaceae (Griffin & Buck, 1989). Inferences from three combined chloroplast loci resolved the genus as sister to the Bryales s.l., but analyses of the *rbc*L or the *trn*L-*trn*F sequences place it in the diplolepideous alternate mosses (Virtanen, 2003). None of these hypotheses is, however, well supported.

No study has yet focused on the infrafamilial relationships within the Funariaceae. Based on a limited sampling, the results by Goffinet and Cox (2000) suggest that, within the Funariaceae, *Funaria* and *Entosthodon* are polyphyletic, and that *Physcomitrella* may not warrant segregation from *Aphanorhegma.* That this family has escaped the attention of molecular systematists is surprising. Indeed, the taxa are easily grown in culture, the complete genome of *Physcomitrella* is being sequenced (Rensing et al., 2002), and the peristome evolution is characterized by severe, but maybe gradual reduction, leading to multiple gymnostomous taxa. Hence, the Funariaceae may be a model system for testing hypotheses on peristome evolution within the diplolepideous mosses, such as the untested assumption that peristome loss may be irreversible.

The circumscription of the Funarialean lineage is still tentative, and the affinities of the Encalyptales and Timmiales are in need of more critical studies. Since increasing the character sampling has not settled the ambiguity (Cox et al., 2004), efforts should perhaps be directed at broadening the species representation, especially in recognition of the phylogenetic significance of gymnostomous taxa (e.g., *Oedipodium* basal to all peristomate mosses).

Phylogeny of the haplolepideous mosses

Mosses with a haplolepideous peristome compose a monophyletic lineage that is well supported by molecular data (La Farge et al., 2000; Newton et al., 2000; Beckert et al., 2001; Tsubota et al., 2003; Werner et al., 2004). The circumscription of this lineage has been extended to accommodate several taxa with reduced peristomes that were previously considered of diplolepideous affinities based on gametophytic features. Goffinet et al. (1998) provided evidence from *rbc*L data that *Drummondia,* the Erpodiaceae, and the Rhachitheciaceae belong to the Dicranineae sensu Vitt (1984) rather than the Orthotrichineae. Their study also led to the transfer of *Amphidium* to the haplolepideous mosses, a hypothesis later supported by Stech (1999a). *Ephemerum, Goniomitrium,* and *Splachnobryum* were also resolved within the haplolepideous clade rather than with the Funarineae based on nuclear or cpDNA data (Goffinet & Cox, 2000). Heddersen et al. (2004) excluded *Bryobartramia* from the Dicranales and suggested that it may be a derived member of the Encalyptaceae. Inferences from nuclear and chloroplast sequences revealed that *Wardia* and *Schistostega* should be aligned within the haplolepideous mosses rather than with the Leucodontineae or Bryineae among the diplolepideous alternate mosses (Cox & Hedderson, 1999; Hedderson et al., 1999; Tsubota et al., 2002). Similarly, *Archidium,* which Vitt (1982, 1984) considered as the basalmost lineage among arthrodontous mosses, has been shown to be instead nested within the haplolepideous mosses (Goffinet & Cox, 2000), although its affinities remain ambiguous (Goffinet et al., 2001). Vitt's (1984) hypotheses that the Buxbaumiaceae and Encalyptaceae are basal lineages within the haplolepideous mosses have been refuted (see discussion above). Similarly, Stech et al. (2003b) inferred the ordinal affinities of *Pulchrinodus* based on cpDNA data and transferred the genus to the diplolepideous mosses, where its relationships remain obscure.

The significance of molecular data resides in its ability to test systematic hypotheses pertaining to the affinities of taxa that are patristically distant from most typical lineages. All phylogenetic reconstructions resolve a group of three genera at the base of the evolutionary tree of the haplolepideous mosses: *Drummondia, Scouleria,* and *Bryoxiphium.* Not only had *Drummondia* been placed by Vitt

(1984) and others in the Orthotrichales, but affinities to neither of the two other genera had ever been proposed. This is hardly surprising considering their highly divergent gametophytes, sporophytes, and even habitats (Vitt, 1982). The precise relationships among the three genera and the remainder of the haplolepideous mosses remain ambiguous (Hax & Goffinet, unpublished), although *Drummondia* and *Scouleria* are often resolved as sister-taxa (e.g., Tsubota et al., 2003). No morphological synapomorphy is readily obvious for any association that involves these genera. It is likely that much of the morphological transformation occurred well after the origin of the taxa and that the synapomorphies have been lost. Although these genera may not be very informative in terms of polarization of morphological character transformations at the base of the haplolepideous mosses, they may be essential for the proper rooting of the core of this lineage.

Six studies have focused exclusively on the phylogeny of the haplolepideous mosses: Stech (1999b), La Farge et al. (2000, 2002), Tsubota et al. (2003), Hedderson et al. (2004), and Werner et al. (2004). In all cases, inferences were made from variation in cpDNA loci. Stech (1999b) reconstructed the relationships based on *trn*L-*trn*F sequences only. Support for the relationships was poor and lacking for the ingroup (i.e., Dicranaceae), subfamilies, and shallower nodes. The remaining studies differ mainly in their taxon sampling because, except for Tsubota et al. (2003), inferences were made at least in part from *rps*4. La Farge et al. inferred the phylogeny also from *trn*L (2000, 2002) and *rbc*L (2000), whereas Tsubota et al. (2003) relied exclusively on the phylogenetic signal contained in the *rbc*L sequences. The relationships among the major lineages are consistently poorly supported, and thus, although these may be correct, further evidence is needed to corroborate the observed phylogenetic pattern. However, these studies resolve three major clades within the haplolepideous mosses. The first one is composed of the core families Dicranaceae, Leucobryaceae, and Calymperaceae, whereas the second includes the Pottiaceae as a crown group, subtended by the Erpodiaceae, and maybe also the Rhachitheciaceae, Ditrichaceae, and Rhabdoweisiaceae. The third lineage comprises the Grimmiales and Seligeriales. The monophyly of the first two main lineages remains to be asserted. In all cases the Seligeriales are sister to the Grimmiales. This hypothesis, which was also proposed by Vitt (1984), is particularly well supported by inferences from *rbc*L sequences (Tsubota et al., 2003). A shared ancestry for the Calymperaceae and Pottiaceae, as proposed by Vitt (1984), is thus not supported by these studies. The phylogenetic significance of the Fissidentaceae within the Dicranales is ambiguous.

Phylogenetic relationships among orders of haplolepideous mosses have never been inferred from morphological characters alone, and the implicit hypothesis proposed by Vitt (1984) is not supported by synapomorphies for sister-groups. Similarly, none of the studies based on molecular data offered morphological transformations that correlate with early cladogenic events. It is likely that major lineages of haplolepideous mosses, and hence of all mosses, have a relative ancient origin that may date back a couple of hundred million years, and any synapomorphies have been erased by subsequent modifications. Considering that mosses are of a rather simple morphology, neither the gametophyte nor the sporophyte offers many characters that could be targeted by selection, and hence adaptive pressures act on a small set of features. However, there is hope that ontogenetic data, such as the protonematal developmental studies carried out by Duckett and his collaborators (see Duckett et al., 2004), may yield characters to support the phylogenetic hypotheses derived from molecular studies for the arthrodontous mosses, similar to those scored by Newton et al. (2000) to complement the sequence data used to reconstruct the phylogeny of all mosses.

The chloroplast loci used by La Farge, Hedderson, and their coauthors provide strong support for several lineages that correspond to families as defined by Vitt (1984), but whose circumscription requires nevertheless some significant amendments. Most notably is the polyphyly of the Dicranaceae sensu Vitt (1984). Following La Farge et al. (2002), the family should include *Wardia* and the Dicnemonaceae, but exclude several other taxa, such as *Campylopus,* which is transferred to the Leucobryaceae, as well as many genera characterized by smaller gametophytes, most of which are best accommodated within the Rhabdoweisiaceae. The circumscription of the Pottiaceae, the only other family specifically targeted (Werner et al., 2002, 2004), underwent fewer changes. The genera included by Vitt (1984) or Zander (1993), except for *Timmiella,* share a common ancestor, but Werner et al. (2004) confirmed that to satisfy a criterion of monophyly, the family must also include the genera transferred earlier by Goffinet and Cox (2000), namely, *Ephemerum, Goniomitrium,* and *Splachnobryum.* Affinity of *Ephemerum* with the Pottiaceae is supported by their shared mode of protonematal development (Duckett et al., 2004). The Seligeriaceae and the

Grimmiaceae appear as monophyletic, whereas the Ptychomitriaceae are resolved by Tsubota et al. (2003) as a polyphyletic group. Based on *rbc*L data, *Glyphomitrium* appears nested within the Rhabdoweisiaceae (Tsubota et al., 2003). Whether *Campylostelium* belongs to the Ptychomitriaceae or Grimmiaceae is not clear (Tsubota et al., 2003).

All the above hypotheses are derived from analyses of chloroplast data. Given that all phylogenetic hypotheses may reflect the evolution of this genome rather than the species sampled, independent testing using nuclear loci is imperative. Spagnuolo et al. (1999) inferred phylogenetic relationships among pottiaceous taxa from variation in the ITS1 (nrDNA) locus, but other ribosomal RNA-encoding genes, which may be better suited for testing hypotheses at suprageneric and familial levels, have not yet been used. Extending the sampling to other loci and genomes may yield the level of variation needed for obtaining robust support for the series of cladogenic events. The speciose families Fissidentaceae and Grimmiaceae have not yet received any attention from molecular systematists.

Phylogeny of the diplolepideous alternate mosses

The diplolepideous alternate peristome is shared by those acrocarpous mosses traditionally defined as the Bryales (Vitt, 1982) or Bryineae (Vitt, 1984), as well as all pleurocarpous lineages (i.e., Hypnidae). Acrocarpous lineages consistently fail to compose a monophyletic lineage that is sister to the pleurocarpous mosses, and one question that has driven much of the interest on this group is to resolve the origin of the pleurocarps (e.g., De Luna et al., 1999, 2000).

The circumscription of the Bryineae sensu Vitt (1982) underwent some revisions in recognition of phylogenetic inferences. The Splachnaceae were considered by Vitt (1984) and earlier workers as allied to the Funariineae or intermediate between it and the Bryineae. Inferences from chloroplast and nuclear data suggest that the Splachnaceae compose a lineage of derived bryalean mosses. The endostome is retained only in the genus *Splachnum*: it lacks cilia, and the segments lay opposite the exostome teeth to which they are fused. This opposite arrangement prompted Vitt (1982, 1984) to consider an early origin of this family. The development of the amphithecial layers is, however, congruent with a bryalean origin (see Goffinet et al., 1999, for an interpretation of the developmental data provided by Schwartz, 1994). The Splachnaceae appear indeed most closely related to the Meesiaceae, including *Leptobryum* based on molecular characters (Cox & Hedderson, 1999; Goffinet et al., 2001).

The Orthotrichaceae sensu Goffinet and Vitt (1998) share a distinct architecture of the peristome wherein the outer peristomial layer is highly thickened and the endostome lacks cilia. These characters formed the basis for Vitt's (1981, 1984) argument to retain the Orthotrichaceae outside of the Bryineae. Phylogenetic evidence for this hypothesis is lacking, as the Orthotrichaceae are consistently resolved in a somewhat nested position near the base of the Bryineae (Cox & Hedderson, 1999; Cox et al., 2000; Newton et al., 2000; Goffinet et al., 2001). Hence, the peristome is better considered as derived from the *Bryum* peristome, as suggested by developmental data (Goffinet et al., 1999). The circumscription of the family has been revised (Goffinet & Vitt, 1998) following the phylogenetic study by Goffinet et al. (1998), which led to the exclusion of several taxa, now accommodated within the haplolepideous or pleurocarpous mosses (Goffinet, 1998; Goffinet & Vitt, 1998).

The Hedwigiaceae contain few species (Crosby et al., 2000). The lack of a peristome precludes an unambiguous supraordinal assignment. The perichaetia can be terminal on stems or branches (Vitt, 1982). Vitt (1982, 1984) placed the family among the pleurocarpous mosses, probably because the leaves are ecostate, a feature extremely rare among acrocarpous arthrodontous mosses. Note that *Bryowijkia,* which has a short costa and a poorly developed peristome, may not belong to this family (Cox, pers. comm.). Inferences for *Hedwigia* support the hypothesis that the family belongs to the diplolepideous alternate mosses, with stronger affinities to the acrocarpous lineages (Goffinet et al., 1998; Cox et al., 2000, 2004; Newton et al., 2000). A nested position within the Orthotrichaceae (De Luna, 1995) is, however, not supported by molecular data (Goffinet et al., 1998; Cox & Hedderson, 1999). The Rhacocarpaceae seem to be closely allied to the Hedwigiaceae (Frahm et al., 1997).

The familial relationships within the Bryineae remain ambiguous. Inferences from one nuclear (18S) and two chloroplast loci (*trn*L-*trn*F, *rps*4) provide strong support for main lineages, some of which corresponded to families, but not for their affinities (Cox & Hedderson, 1999). Broadening the character (*rbc*L) and taxon sampling did not improve the level of confidence with regard to individual nodes (Cox et al., 2000). From these studies *Orthodontium* emerges as the acrocarpous Bryalean moss most closely related to the pleurocarps. Multigenomic inferences, based on eight loci for a restricted sampling of taxa, yielded little support for

this hypothesis (Cox et al., 2004), but this may be due to (1) low taxon sampling among Bryalean mosses, and (2) exclusion of potential informative regions due to ambiguous homology of DNA regions across all mosses.

Although the Bryineae are characterized by their acrocarpous development of perichaetia, several families exhibit perichaetia produced on short lateral branches. The Spiridentaceae, Hypnodendraceae, Pterobryellaceae, Cyrtopodaceae, Hypopterygiaceae, and Racopilaceae all have creeping stems and lateral sporophytes (Vitt, 1982), yet their position in classifications is inconsistent. Vitt (1982, 1984) and Buck and Vitt (1986) regarded the Spiridentaceae as a member of the Bryineae or its equivalent taxon. The Hypnodendraceae were placed in the Bryineae or Bryales by Vitt (1984) and Vitt (1982), respectively, but then moved to the Hypnales (Buck & Vitt, 1986). The Pterobryellaceae were treated as Leucodontalean taxa by Vitt (1982, 1984), but with Hypnalean affinities by Buck and Vitt (1986). The Cyrtopodaceae were also considered pleurocarpous mosses and placed in the Leucodontales (or its equivalent; Vitt 1982, 1984; Buck & Vitt, 1986). Vitt (1982, 1984) placed the Racopilaceae in the Hypnales, but Buck and Vitt (1986) regarded the family to have a Bryalean origin and to be closely related to the Hypopterygiaceae. The latter was placed in the Hookeriales by Vitt (1982, 1984) before being transferred to the Bryales by Buck and Vitt (1986). Inferences from chloroplast loci have shown that all these families, with the exception of the Hypopterygiaceae, are not nested within the main lineage of pleurocarps and are instead of Bryalean origin (Withey, 1996; De Luna et al., 1999; Buck et al., 2000; Goffinet et al., 2001). Whether this lineage of Bryalean pleurocarps may be a sister-group to the true pleurocarps and hence share a common ancestor and thus be regarded as an early derivation within the pleurocarps is not fully resolved (Buck et al., 2000; De Luna et al., 2000; Tsubota et al., 2002). Inference from *rbc*L sequences are incongruent with that hypothesis unless they are combined with morphological data (De Luna et al., 1999). The Hypopterygiaceae are consistently resolved as a sister to the Hookeriales by these studies (Buck et al., 2000), and this hypothesis is corroborated by nuclear and mitochondrial data (Buck et al., 2004). The affinities of the other families to the Bryales raise the question about possible multiple origins of pleurocarpy. La Farge-England (1996) considered all these families (except the Pterobryellaceae, which she did not study) to be pleurocarpous based on her stringent definition of pleurocarpy. Pleurocarpy would thereby have arisen twice. In neither clade is there evidence of reverse evolution and pleurocarpy appears to result from an irreversible transformation.

DNA sequence data have also provided the basis to test generic circumscription and relationships within the Bryineae sensu Vitt (1982) or Bryales (cf. Appendix 1). The Splachnaceae are best known for some of its species that occur on substrates of animal origin and that rely on insects to disperse their spores. Much of the classification of the family was based on sporophytic characters, of which many could be considered linked to the dispersal strategy. Inferences from cpDNA suggest that several supraspecific concepts did not satisfy a criterion of monophyly (Goffinet & Shaw, 2002; Goffinet et al., 2004a). *Brachymitrion* Taylor was placed in synonymy with *Tayloria,* and the Voitioideae were transferred to the Splachnoideae within the Splachnaceae. Lineages resolved as monophyletic based on DNA evidence do, however, lack any non-homoplasious morphological synapomorphy.

Within the core clade of Bryalean mosses, Cox and Hedderson (1999) have further shown that the Bryaceae are polyphyletic, and that the genera around *Pohlia* form a monophyletic lineage within the Mniaceae. This and other studies (Cox & Hedderson, 2003; Pederson & Hedenäs, 2003; Pedersen et al., 2003) have further shown that the speciose genus *Bryum* is polyphyletic if some of its segregates are considered distinct at the generic level. Modifications of the peristome may define monophyletic entities, but the taxa with the ancestral state compose a grade in the Bryaceae. Hence, peristomial architecture is not a reliable phylogenetic indicator. *Orthodontium* is consistently resolved outside of the Bryaceae and seems to be more closely related to the immediate ancestor to the pleurocarps (Cox & Hedderson, 1999; Cox et al., 2000; Goffinet et al., 2001), although its precise affinities remain ambiguous (Cox et al., 2004).

Reconstructions within the Orthotrichaceae (Goffinet al., 1998) revealed the polyphyly or paraphyly of several taxa (e.g., the Zygodontoideae, *Zygodon* and *Orthotrichum*) and some of these observations have been integrated into a new classification (Goffinet & Vitt, 1998). Analyses of nuclear and mitochondrial loci have corroborated the earlier conclusions and further systematic changes have been made (Goffinet et al., 2004b). From these it appears, as is the case of the Amblystegiaceae (see section on pleurocarp phylogeny), that neither sporophytic nor gametophytic characters are consistently informative about relationships across the family. The phylogeny inferred from molecular data

is consistent with the gametophytic differentiation of *Codonoblepharon* and *Leratia* and supports the rejection of segregate genera solely on the basis of sporophytic characters (e.g., *Bryodixonia* Sainsbury, *Muelleriella* Dusén, and *Leptodontiopsis* Broth.). Furthermore, the morphological similarities between *Orthotrichum exiguum* Sull., *Bryomaltaea* Goffinet, and *Leratia* are shown to result from shared ancestry rather than convergent evolution; hence, all three taxa are now accommodated within *Leratia* (Goffinet et al., 2004b).

Among the diplolepideous mosses, the Bartramiales sensu Vitt (1982) appears as a somewhat isolated lineage due to its globose capsules and the typically prorulose cells. The Bartramiaceae are the only family whose circumscription has remained stable following phylogenetic investigations (Virtanen, 2003), although she confirmed that *Catoscopium*, which had been transferred to the Bartramiaceae by Griffin and Buck (1989), should be excluded from the family as suggested by the results of Goffinet et al. (2001). The genera *Bartramia* and *Philonotis* appear paraphyletic, but overall support is weak to moderate, even if morphological data are added to the sequences (Virtanen, 2003).

The contribution of molecular data to the systematics of the Bryineae sensu Vitt (1984) lay particularly in the recognition that the acrocarpous mosses with a diplolepideous alternate peristome compose a paraphyletic assemblage that includes the Splachnineae, Orthotrichineae, and all the so-called Bryalean pleurocarps, and that the evolution of this grade leads to the pleurocarpous mosses. Although the loci sampled so far help define the circumscription of the families and hence to identify natural lineages, much further work is needed to determine the sequence of cladogenesis within this grade, and in particular the sister-group to the pleurocarps.

Circumscription of pleurocarpous mosses

Perichaetia arising from lateral buds rather than at the apex of differentiated branches and stems characterize the pleurocarps. As discussed above, pleurocarpy per se does not suffice to define this clade. Hedenäs (1994) suggested that this clade may be better diagnosed by a homogeneous costal anatomy. However, this character too may not be unique to pleurocarps, and perhaps the clade is best defined by a combination of both characters.

Although long seen as a natural lineage, this clade lacked a name until Buck et al. (2000) erected the Hypnidae to accommodate them. The monophyly of the Hypnidae is well supported by molecular data (e.g., Tsubota et al., 2002). Three main lineages of pleurocarpous mosses have traditionally been recognized based among other characters on peristomial architecture and type of modular growth or branching (Buck & Vitt, 1986): the Hypnineae (or Hypnobryales sensu Vitt, 1982), the Leucodontineae (or Isobryales sensu Vitt, 1982), and the Hookeriineae (or Hookeriales sensu Vitt, 1982). The Hypnineae were defined by "specialized sporophytic features" (Buck & Vitt, 1986: 39), including asymmetric, horizontal capsules whose mouth is lined by a distinctly ornamented peristome characterized by the abruptly tapered exostome teeth (see Buck & Vitt, 1986, for details). This lineage is predominant in north temperate climates. The Leucodontineae were diagnosed by their creeping, sympodially branching stems and by erect capsules with gradually acuminate and poorly ornamented teeth. The Hookeriales also have similar sporophytes but their exothecial cells are collenchymatous, and their endostome segments have a bamboo-like architecture (Buck, 1998). Whether these morphological characters serve to identify monophyletic groups was first investigated by Buck et al. (2000) and De Luna et al. (2000). Both of these studies clearly showed that the pleurocarps compose two main lineages, equivalent to the Hookeriales and the Hypnales (including the Leucodontales). Indeed, neither the Hypnales nor the Leucodontales sensu Vitt (1982) are monophyletic. Instead the two orders are intimately mixed as is shown also by inferences from *rbc*L sequences alone (Tsubota et al., 2002). Consequently, none of the characters seen as diagnostic of the two orders is a good phylogenetic indicator. Reduction of the peristome and a shift to a sympodial mode of branching have occurred multiple times and likely parallel a transition to epiphytism (Buck et al., 2000), a hypothesis formulated earlier by Buck (1991).

The Hookeriales are monophyletic if the Hypopterygiaceae are included. Blöcher and Capesius (2002) refuted an affinity of the Hypopterygiaceae to the Hookeriales based on *rps*4 sequences (which were also used by Buck et al., 2000). Support for an exclusion of this family from the Hookeriales-Hypnales clades was, however, lacking. The Ptychomniaceae and Garovagliaceae have traditionally been placed in the Leucodontineae (Vitt, 1982 [as "Leucodontales"], 1984), but rather the group has been resolved as a sister-group to the combined Hookeriales-Hypnales by nuclear and mitochondrial data (Buck et al., 2004). The later authors have now synonymized the two families and accommo-

dated the Ptychomniaceae within its own order the Ptychomniales, sole order of the Ptychomnianae.

The Hookeriales are now interpreted as consisting of seven families (Buck et al., 2004). The order may have undergone a constant rate of diversification, and although it is much less diverse than its sister group, the Hypnales, the Hookeriales contain much larger amounts of phylogenetic diversity (Shaw et al., 2003b).

Although much effort has been invested in resolving the relationships among pleurocarpous families, only those involving the Hookeriales are well supported (Buck et al., 2004). Within the Hypnales, phylogenetic inferences have relied mostly on cpDNA sequences (i.e., *rbc*L, *rps*4, and *trn*L-*trn*F), except for the studies on the Amblystegiaceae (Vanderpoorten et al., 2001, 2002a) or Lembophyllaceae (Quandt et al., 2000). Families often receive moderate support (except in Goffinet et al., 2001, where it is virtually lacking), but inferences regarding their mutual relationships are rarely robust (e.g., Tsubota et al., 1999, 2002; Buck et al., 2000; Maeda et al., 2000; Arikawa & Higuchi, 2002). Evolutionary histories have been reconstructed based on DNA sequences for the following families: Amblystegiaceae (Vanderpoorten et al., 2001, 2002a, b), Fontinalaceae (Shaw & Allen, 2000), Hylocomiaceae (Chiang & Schaal 1999a, b, 2000a, b), Hypnaceae (Tsubota et al., 1999; Arikawa & Higuchi, 2003), Lembophyllaceae (Quandt et al., 2000), Plagiotheciaceae (Arikawa & Higuchi, 1999, 2002), and Sematophyllaceae (Tsubota et al., 2000, 2001a; Akiyama & Tsubota, 2001).

The Hypnaceae are consistently resolved as a polyphyletic entity (Tsubota et al., 1999, 2002; Buck et al., 2000). For example, *Platygyrium* is resolved within the Sematophyllaceae based on *rbc*L data (Arikawa & Higuchi, 2003). The inferences based on multiple genomic sequences also suggest that a shift from a single to a double costa has occurred multiple times (Tsubota et al., 1999; Buck et al., 2004) and hence that a reduction of the single costa is not a reliable phylogenetic indicator as proposed by Buck and Vitt (1986). The polyphyly of the Hypnaceae is also accounted for by the polyphyly of *Hypnum* itself. Tsubota et al. (1999, 2002) confirmed the hypothesis proposed by Hedenäs (1990) based on morphological characters that *H. lindbergii* Mitt. is more closely related to *Calliergonella* (Amblystegiaceae) than to other species of *Hypnum* (note, however, that *Calliergonella* is excluded by Vanderpoorten et al. (2002b) from the Amblystegiaceae). *Hypnum tristo-viride* (Broth.) Paris is, by contrast, closely related to *Brotherella* (Sematophyllaceae for Tsubota), whereas *H. cupressiforme* Hedw., the type of the genus, perhaps is sister to the Entodontaceae–Sematophyllaceae clade (Tsubota et al., 2000), but this relationship remains dubious (Tsubota et al., 2001a, b).

Several studies have focused on the circumscription of the Sematophyllaceae (Tsubota et al., 2000, 2001a, 2001b). Tsubota et al. (2000) confirmed the inclusion of *Brotherella* and related genera in this family rather than in the Hypnaceae. Similarly, *Isopterygium* was regarded as a member of the Sematophyllaceae based on *rbc*L data (Tsubota et al., 2001a). By contrast, *Glossadelphus* was excluded from the family and considered a possible member of the Hypnaceae (Tsubota et al., 2001a). The affinities of *Taxithelium* remained unresolved (Tsubota et al., 2001a, b), although when a smaller set of hypnoid taxa was analyzed the genus fell within the Sematophyllaceae (Akiyama & Tsubota, 2001). The unstable circumscription of the Hypnaceae and Sematophyllaceae reveals that none of the characters used historically serves as a diagnostic feature of either family, or that these characters (e.g., strongly shouldered exostome for the Sematophyllaceae (Buck & Vitt, 1986)) have undergone further modifications during the evolution of the family. Hedenäs and Buck (1999) reconstructed the phylogeny of the Sematophyllaceae from variation in morphological characters. Constraining their inferences to the phylogenetic hypothesis inferred from *rbc*L sequences may yield the synapomorphies needed to diagnose the Sematophyllaceae as redefined by Tsubota et al. (2001a). The value of morphological characters for defining genera within the family has also been questioned since several genera (i.e., *Trismegistia*, *Mastopoma*, and *Wijkia*) may compose polyphyletic entities (Akiyama & Tsubota, 2001; Tsubota et al., 2001b). In the classification system presented below, we have made an initial attempt to help resolve the problems with a polyphyletic Sematophyllaceae by describing the new family Pylaisiadelphaceae for a number of these more Hypnoid-like genera.

The brown mosses or Amblystegiaceae typically grow in wet habitats. A phylogenetic reconstruction of pleurocaprous mosses based on two chloroplast markers led Buck et al. (2000) to suggest that *Anacamptodon* should be included in this family rather than the Fabroniaceae (Vitt, 1984), a hypothesis corroborated by inferences from ITS, *trn*L-*trn*F, and *atp*B-*rbc*L intergenic spacer and morphology (Vanderpoorten et al., 2002a). The latter study also indicated that the Amblystegiaceae, as defined by Vitt (1984) and older classifications, are polyphyletic. The genera form two main lineages and Vanderpoorten et al. (2002b) treat these as two families:

the Amblystegiaceae, accommodating 14 genera, and the Calliergonaceae, which are defined by inflated alar cells, comprising 5 genera. The families do not share a most recent common ancestor, but their respective sister group is still unknown. *Donrichardsia* and *Platyhypnidium* are transferred to the Brachytheciaceae (Stech & Frahm, 1999), and *Platylomella* is also consistently resolved outside the family, but remains of uncertain affinities (Arikawa & Higuchi, 1999; Tsubota et al., 2002). Similarly, the affinities of *Conardia*, *Campylophyllum*, and several species of *Hygrohypnum* (other than the type species) remained uncertain, except that they clearly appeared unrelated to the Amblystegiaceae s. str. (Vanderpoorten et al., 2002b). The former two genera may be best accommodated in the Hypnaceae (cf. Appendix 1). Ancestral character reconstructions based on their optimal phylogeny reveal that (1) transformations in both sporophytic and gametophytic characters are correlated to shifts in habitat (unless these characters are genetically linked to characters that are adaptive to the habitat), and (2) that these characters are more labile than previously thought (Vanderpoorten et al., 2002a). Broad pseudoparaphyllia, for example, have likely arisen four times in taxa that were previously treated as members of the Amblystegiaceae sensu Vitt (1984), but are now considered unrelated (Vanderpoorten et al., 2002a). Although the lability of the characters has not decreased following the phylogenetic studies by Vanderpoorten et al. (2002a), they tend to be more stable within the new defined clades (Vanderpoorten et al., 2002a, 2002b). The circumscription of the Amblystegiaceae, as amended by these authors, leaves their Amblystegiaceae without a single morphological synapomorphy.

The Hylocomiaceae are among the most conspicuous mosses in boreal to subarctic regions. The delimitation of the family and its separation from the Rhytidiaceae is controversial. Buck (1980) excluded *Rhytidium* but included *Pleurozium*. Based on inferences from ITS2 and *atp*B-*rbc*L spacer sequences (Chiang & Schaal, 2000a, 2000b), *Rhytidium* appears to be nested within the Hylocomiaceae, whereas *Pleurozium* is sister to it. However, it is not clear whether *Pleurozium* should be included or not in the Hylocomiaceae, since it is drawn to the single outgroup species (i.e., *Entodon seductrix* (Hedw.) Müll. Hal., Entodontaceae). Additional outgroup taxa are needed to test whether the 94% bootstrap value given based on ITS2 (Chiang & Schaal, 2000b) characterizes a sister-group association of *Entodon* and *Pleurozium* versus a shared ancestry of *Pleurozium* and the remaining Hylocomiaceae. An unusual peristome ornamentation in *Pleurozium* seems to be found otherwise only within the Hylocomiaceae, and similarly the chromosome number of $n = 4$ is strongly indicative of a relationship to that family (Buck, 1980). The studies by Chiang and Schaal (2000a, b) also resolve the monogeneric Theliaceae as a member of the Hylocomiaceae. Such phylogenetic affinities for the Theliaceae have not been proposed previously. Although this hypothesis is well supported by both sources of molecular characters, it remains to be tested within a broader context of pleurocarp phylogeny to determine whether this association is an artifact due to the limited sampling. *Rps*4 data do not resolve the Hylocomiaceae as monophyletic (based on two genera only; De Luna et al., 2000; Goffinet et al., 2001). Support for the monophyly is provided by *rbc*L (Tsubota et al., 2002), but the former locus has not been sequenced for *Thelia*, and hence the affinities of this genus, and thus of the Theliaceae to the Hylocomiaceae, remain ambiguous. The study by Tsubota et al. (2002) also resolves *Ctenidium*, a genus traditionally aligned with the Hypnaceae, as a member of the Hylocomiaceae, based on *rbc*L data. This hypothesis, too, remains to be critically tested.

The circumscription of the Lembophyllaceae has been revised based on variation in cpDNA and nrDNA sequences (Quandt et al., 2000). The phylogenetic inferences support the retention of *Weymouthia* in the Lembophyllaceae rather than in the Meteoriaceae, and indicate further that the definition of the later is in need of further research, considering the ambiguous affinity of *Pilotrichella* to either of these families based on *trn*L and ITS data. Akiyama and Tsubota (2004) also argued for the polyphyly of the Lembophyllaceae. Their study lends support to Buck's (1980) hypothesis that *Dixonia* should be excluded from this family, but the alternative affinities of this genus remain unclear. The genus might be better accommodated within the Neckeraceae.

The Brachytheciaceae are one of the most speciose families of pleurocarpous mosses (cf. 43 genera, Appendix 1), and also one exhibiting the widest range of morphological variation. Hence, it is not surprising that generic circumscriptions and relationships have been controversial (Ignatov & Huttunen, 2002). Huttunen and Ignatov (2004) tested phylogenetic hypotheses within this family by combining sequence data from two chloroplast regions (*trn*L-*trn*F and *psb*T-*psb*N-*psb*H regions) and one nuclear locus (ITS2) with morphological characters. Their inferences resolved the Brachytheciaceae as essentially defined by Buck and Goffinet (2000) as

monophyletic (with minor generic alterations), and sister to the Meteoriaceae. The phylogenetic hypotheses served as a basis for the subsequent systematic interpretation and a new classification of the Brachytheciaceae (Ignatov & Huttunen, 2002). The chloroplast loci exhibit little variation to resolve infrageneric relationships, whereas the nuclear ITS2 region may, in some cases, be too variable to even resolve species as monophyletic entities. Independent analyses of the four character partitions yielded different topologies, but the incongruence was not significant enough and character sources were combined. To what extend the optimal topologies retrieved from the combined data set are shaped by the morphological data is not clear, although it was noted that the total-evidence phylogeny mostly resembled the topologies obtained from analyzing the ITS2 data alone.

The monophyly of the Brachytheciaceae sensu Ignatov (1999) is well supported. The family is now best defined by the arrangement and shape of the pseudoparaphyllia and the smooth laminal cells, although exceptions within and outside the family defy this generalization. Four main lineages are now recognized as subfamilies within the Brachytheciaceae (Ignatov & Huttunen, 2002). Support for the monophyly of these clades varies (Huttunen & Ignatov, 2004): it is high for the Homalothecioideae but low to absent for the other three subfamilies. In the case of the Rhynchostegioideae monophyly is dependent on the equal weighting of the morphological data, and, even then, support values are virtually lacking. Several speciose genera (e.g., *Eurhynchium* s.l.) are revealed as polyphyletic, and others are resolved only as monophyletic lineages if morphological characters are included in the analyses (e.g., *Kindbergia*). Peristome reduction is shown to have occurred in all clades. Except for the Rhynchostegioideae, the subfamilies are shown to be polymorphic for the roughness of the seta (smooth vs. rough), and all but the Rhynchostegielloideae are variable with regard to the shape of the operculum. These and other characters have traditionally been used to define supraspecific taxa within the family, and are shown to be broadly homoplasic. Hence, as has been the case in other families (e.g., Splachnaceae), many clades resolved within the Brachytheciaceae by the phylogenetic analyses cannot be diagnosed by "known morphological characters" (Huttunen & Ignatov, 2004: 175). Much remains to be done to elucidate the generic definitions and relationships within the Brachytheciaceae, but this study offers a sound basis for further research.

The Plagiotheciaceae sensu Vitt (1984) are resolved as a polyphyletic entity with *Entodontopsis* and *Stereophyllum* forming a monophyletic lineage distantly, but also ambiguously related to the *Plagiothecium* as suggested by Buck and Ireland (1985), who erected the Stereophyllaceae to accommodate *Entodontopsis* and *Stereophyllum* as well as other genera. The inclusion of *Herzogiella* in the Plagiotheciaceae as proposed by Pedersen and Hedenäs (2001) is not well supported (Arikawa & Higuchi, 2003), but neither is its exclusion (Arikawa & Higuchi, 2002).

A series of studies have dealt with the status of species-poor or often monospecific genera erected for aquatic mosses that are characterized by a pluristratose lamina and/or leaf border and a broad costa. These features are otherwise very rare among pleurocarpous mosses. Furthermore, most of these taxa are sterile (except *Hypnobartlettia* and *Platyhypnidium mutatum* Ochyra & Vanderpoorten), and hence their generic or familial affinities are ambiguous. Molecular data have proven essential to settle the controversy regarding the systematic status of these aquatic mosses. *Donrichardsia, Platyhypnidium riparioides* (Hedw.) Dixon, and *P. mutatum* are now considered members of the Brachytheciaceae (Stech & Frahm, 1999); *Gradsteinia* and *Ochyraea* were transferred to the Amblystegiaceae (Stech & Frahm, 2000, 2001). Affinities of *Ochyraea* to the Amblystegiaceae were based on its relationships to *Calliergonella* and *Hygrohypnum*, which were subsequently excluded from the family (Vanderpoorten et al. 2002b). *Ochyraea* is now accommodated in the Hypnaceae (Vanderpoorten et al., 2002b). The Hypnobartlettiaceae and Vittiaceae were merged with the Amblystegiaceae (Vanderpoorten et al., 2002b, 2003). These inferences have provided the first molecular evidence of convergent evolution in gametophytic characters in mosses in response to shifts in habitats. Whether these modifications are genetically fixed or phenotypic expressions has not been formally tested. In other taxa, morphological characters appear to be very much shaped by environmental parameters. For example, all morphological species centered around *Amblystegium tenax* (Hedw.) C. E. O. Jensen are now considered to merely represent phenotypic variants (Vanderpoorten, 2004). A similar situation may occur in *Fontinalis*. Shaw and Allen (2000) sampled 13 taxa within *Fontinalis*, and several were represented by multiple populations distributed across their transatlantic ranges. Inferences from ITS and *trn*L-*trn*F sequences revealed that all but one of the morphological species for which two or more samples were included are not monophyletic, and the only one representing a natural

lineage (i.e., *F. squamosa* Hedw.) is nested in a clade of *F. antipyretica* Hedw. Hence, characters of the leaves used to define species of *Fontinalis* appear particularly labile in this group of aquatic mosses.

Testing systematic concepts developed by Vitt (1984) or Buck and Vitt (1986) against phylogenetic inferences based on morphological characters revealed that many suprageneric taxa did not satisfy a monophyly criterion (e.g., Hedenäs, 1995; Hedenäs & Buck, 1999). The contribution of molecular data has been particularly significant in addressing the affinities of taxa with reduced morphologies and taxa that have undergone severe morphological transformations in response to a shift in habitat. The phylogenetic association of taxa occupying different habitats, and hence with distinct morphological adaptations, has not solved the problem of the morphological heterogeneity of families. Hence, some families cannot be diagnosed by a single morphological synapomorphy that is conserved by all its taxa.

Although analyses of DNA sequence data have led to a revision of most familial circumscriptions and have offered moderate to strong support for the monophyly of these clades, the relationships among families of pleurocarpous mosses remain, for the most part, ambiguous. The difficulty in resolving the evolutionary history of the Hypnidae may be a direct consequence of their rapid radiation (Shaw et al., 2003b) and hence the paucity of fixed changes in the ancestor to diverging families. The problem is compounded by the fact that many exemplars may not be representative of their family (since many are shown to be polyphyletic) or even their genus. Studies need to include a broad systematic spectrum because many taxa may not be monophyletic, but, of course, by doing so and by focusing on a single locus, homoplasy is potentially rampant and the prospect of resolving the relationships rapidly vanishes. De Luna et al. (2000) showed that for a limited number of pleurocarps resolution is dramatically increased as the number of characters increases. However, the combination of three chloroplast loci failed to offer much support for the nodes of the better resolved consensus tree obtained for hypnobryalean mosses.

Most inferences rely on variation in chloroplast loci, and few have included sequences of the nuclear genome. Variation in the 18S locus is minimal (Capesius & Stech, 1997). Buck et al. (2004) inferred relationships in the Hookeriales in part from the 26S locus. Whether phylogenetic informative variation could be retrieved for the Hypnales from this locus is not clear, especially in the light of the evidence of a rapid radiation of this lineage (Shaw et al., 2003b). The ITS region has been used to assess relationships among species of the liverwort genus *Plagiochila* (Dumort.) Dumort., as well as among populations (e.g., *Mielichhoferia*; Shaw, 2000a), species (e.g., *Fontinalis*; Shaw & Allen, 2000), and genera of acrocarpous (e.g., Pottiaceae; Spagnuolo et al., 1996, 1999) and pleurocarpous mosses (Brachytheciaceae; Huttunen & Igantov, 2004). Among pleurocarpous mosses, this region may be useful to reconstruct the relationships among genera (e.g., Quandt et al., 2000; Vanderpoorten et al., 2001; Huttunen & Ignatov, 2004). Whether the ITS region can contribute to resolving the ambiguity characterizing the relationships among most families of pleurocarpous mosses remains to be tested.

Perspectives

Phylogenetic inferences based on DNA sequence data have shed light on the circumscription of and relationships among various taxa of mosses, from orders to genera and species. Molecular phylogenetics has corroborated many core familial concepts, but the circumscription of virtually all families examined so far must be revised. In many cases, these revisions implicate taxa that are heterogeneous, and hence for which diagnostic characters (i.e., synapomorphies) cannot be defined. Considering that the molecular tools have only been applied recently to bryophyte systematics, it is not surprising that many basic hypotheses remain to be tested in addition to the many that have been raised by recent investigations. Efforts invested in resolving either the supraordinal phylogeny of mosses or the relationships among lineages within the Hypnidae have remained poorly rewarded, as little progress has been made at these levels.

Most inferences rely on variation in chloroplast loci, with fewer studies sampling the nuclear or mitochondrial genome. Few chloroplast loci have been sampled beyond the traditional ones (*trn*L-*trn*F, *rps*4, *rbc*L, and, to a lesser extent, the *atp*B-*rbc*L spacer), but these may merit more attention. Shaw et al. (2003a) and Pedersen and Hedenäs (2003) sequenced *psb*A, and the former study also included *psb*T. Variation in *psb*A among major lineages of Sphagnaceae and Bryaceae was higher than that found for *rps*4 in both studies and similar to those observed for *rbc*L. *Psb*T was more conserved than either of these loci (Shaw et al., 2003a). *Rpl*16 is a protein-coding gene that is slightly more variable than the *psb*A locus (Pedersen & Hedenäs, 2003). The *mat*K region, which has been used extensively

by phanerogamists (Hilu & Liang, 1997; Hilu et al., 2003), has not yet been sampled for bryophytes, although it is currently being screened for addressing the phylogeny of the Pottiaceae (Quandt, pers. comm.). The divergence in the *chl*B sequences among land plants is higher than that of the *rbc*L gene, as well as in mosses and liverworts (two taxa of each sampled; Boivin et al., 1996), and suggests that this locus may be informative at ordinal or higher levels. Primers for the *trn*G locus were developed by Pacak and Szweykowska-Kulinska (2000) and used by Pedersen and Hedenäs (2003). The level of variation exceeded that of several chloroplast loci and hence appears promising as a source of characters.

Following the pioneering work by Beckert et al. (1999), the *nad*5 locus has been used to reconstruct the relationships among major lineages of mosses (Cox et al., 2004) as well as orders among pleurocarpous orders (Buck et al., 2004). *Nad*2 exhibits a similar level of variation (Beckert et al., 2001). Cox et al. (2004) also used *nad*7. *Nad*5 and *nad*7 were sampled by Shaw et al. (2003a) for the Sphagnaceae, which showed about 10% phylogenetically informative sites. By contrast, the level of divergence in the *cox*3 gene between *Ceratodon* and *Physcomitrella* is low (Malek et al., 1996), which may explain why systematists have not yet targeted this locus.

The nuclear genome has offered the least number of loci used so far for phylogenetic reconstruction in bryophytes. The nrDNA repeat is the most widely used nuclear character source for inferring phylogenies of angiosperms (Hershkovitz et al., 1999), as well as for bryophytes. Besides the rRNA encoding genes and the spacers present in the repeat units, few loci have been sequenced. Wall (2002) inferred relationships within *Mitthyridium* (Calymperaceae) based on glyceraldehyde 3-phosphate dehydrogenase sequences. Although the primers developed for this study are presented as being universal, at least within mosses, no other study has used this locus, and our own attempts with these primers have failed to yield any PCR product. Vanderpoorten et al. (2004) targeted the gene for the adenosine kinase enzyme for the *Amblystegium* complex, but obtained multiple amplicons due to the presence of paralogous loci. Similarly, several genes may be present in *Ceratodon* (Shaw et al., 2002), whereas in *Physcomitrella* a single locus occurs (von Schwartzenberg et al., 1998). Shaw et al. (2002) also reported on two other nuclear loci (Δ^6-fatty acyl acetylenase/desaturase (Sperling et al., 2000), and a phytochrome (Pasentsis et al., 1998); all three loci are being sequenced for populations of *Ceratodon purpureus* (Hedw.) Brid. The results of these studies are not yet published, and it is not clear whether the signal is suitable for phylogenetic as well as phylogeographic studies. Sequences of the RNA polymerase II (Denton et al., 1998) and a phytochrome (Kolukisaoglu et al., 1993) have been published and examined for their levels of variation among land plants, but have not been surveyed further within the context of bryophyte phylogeny. Similarly, a family of cytosolic small heat-shock proteins has been characterized for mosses (Waters & Vierling, 1999), but not explored for its potential in phylogenetic inferences.

Shaw et al. (2003a) searched for new sources of phylogenetic characters by exploring the suitability of randomly amplified loci. They found two regions, of unknown genomic homology, whose level of variation was adequate for resolving the sectional phylogeny within *Sphagnum*. Bailey et al. (2004) applied a similar strategy for uncovering loci that display mutations between closely related angiosperm species (i.e., species of *Leucaena*, Fabaceae). The two RAPD loci they ultimately selected exhibited levels of variation that were higher than those observed for cpDNA restriction sites or the ITS region, and led to greater phylogenetic resolution and higher bootstrap values. Screening of amplicons obtained by using RAPD primers may thus offer access to the vast portions of the genome that have remained largely untouched by systematists.

In the past, phylogenetic inferences were based on a single locus (e.g., Goffinet et al., 1998), but increasingly the trend is to combine data from several loci (e.g., Pedersen & Hedenäs, 2003) and ultimately from several genomic compartments (e.g., Shaw et al., 2003a; Buck et al., 2004). Inferences from single loci should be avoided, in part because the level of resolution and support may be low (i.e., Goffinet et al., 2001), or because lateral gene transfer (e.g., hybridization) is potentially more common in mosses than previously thought (e.g., Shaw & Goffinet, 2000; Såstad et al., 2001). Of course, the debate over more taxa (e.g., Tsubota et al., 2002) or more characters (e.g., Cox et al., 2004) is unsettled. If the ambiguity must be settled, one has to choose a criterion upon which to favor one approach over the other. Most often support values are taken as an indicator of accurateness of the phylogeny (as is done throughout this review). However, low bootstrap percentages or low posterior probabilities reveal only that the support is lacking, but not that the hypothesis is incorrect. A hypothesis can only be rejected if an alternative scenario can be significantly favored. For example, the relationships inferred from the small *rps*4 gene based

on 225 species of mosses (Goffinet et al., 2001) are mostly not well supported, but many of the unsupported branches are likely correct (e.g., monophyly of the Splachnaceae, monophyly of the pleurocarps). Long branch attraction (Felsenstein, 1978) has often been invoked to account for results that are in conflict with morphology (e.g., Hyvönen et al., 2004). When mutations yield the same nucleotide in several positions in unrelated taxa, phylogenetic inferences may lead to these taxa being attracted to each other. Thus, the convergence outweighs the true synapomorphies that would place these taxa in their respective clades. It seems clear, without much demonstration, that this can be avoided when species allied to the taxa in question are added, as they would break down the attraction caused by convergence in other sites. Thus, single locus phylogeny for a large taxon sampling may be acceptable, but not desirable. What must be avoided are inferences on a small set of taxa for which a single gene is targeted. Similarly, studies testing the circumscription of an ingroup should include more than one outgroup to avoid the pitfall of a priori bias.

The underlying causes to the phylogenetic ambiguity lay not only with the paucity of the loci sampled. In some cases, the incongruence between studies could be traced to a misidentified herbarium collection (e.g., *Mittenia* in Goffinet et al., 2001; see Bell & Newton, 2004) or culture collection (Arikawa & Higuchi, 2003). A lack of care also resulted in Goffinet (in Goffinet et al., 2001) moving *Pleurophascum* to the Bryales (Buck & Goffinet, 2000), when a closer look at the matrix should have revealed that the *rps*4 sequence for *Pleurophascum* was incomplete. Fortunately, the vast majority of studies are rigorous in confirming the identity of the exemplars that are sampled.

The incongruence between the phylogenetic signals of morphological and molecular data is often implicitly attributed to erroneous homology assumptions of morphological characters. Although the homology of individual sequence characters can also be questioned, especially for non-coding regions that vary in length, a more fundamental assumption, namely, the orthology of the sequences compared between taxa is often implicitly made but rarely tested. Vanderpoorten et al. (2004) revealed extensive duplication of the locus encoding the adenokinase gene in *Hygroamblystegium*. Similarly, the ITS region, which is widely used for its variation among species or populations, occurs in numerous copies (Hillis & Dixon, 1991), and some of these may be pseudogenes (Bailey et al., 2003), which may have their own phylogenetic utility (Razafimandimbison et al., 2004). Clearly, great care must be taken to assert the homology of the loci targeted for phylogenetic reconstructions.

The phylogenetic signal contained in a set of sequences can be retrieved under different assumptions. The sequences of most loci, including those that do not code for a protein or an RNA molecule, may only vary within a space constrained by the secondary structure of either the transcribed product or the ultimate translated product. Many analyses under maximum parsimony treat all characters and all transformations within them as equally probable and equally costly. Inferences under maximum likelihood tend to integrate different rates of evolution for discrete character partitions, as well as different costs for particular nucleotide substitutions. It is beyond the scope of this review to enter the debate regarding the pros and cons of various optimality criteria. However, Cox et al. (2004) showed that, at least at deep levels of the phylogenetic tree (toward the root), saturation of the protein-coding genes may lead to long branch attraction. This problem is alleviated by replacing the third codon position bases by a binary character (purine-pyrimidine) which downweighs transitions or by invoking a model of sequence evolution for the partitions (i.e., 1st, 2nd, and 3rd codon positions; Cox et al., 2004). The secondary structure of non-coding regions should also be taken into account when defining the character homology assumptions (i.e., aligning the sequences; An et al., 1999; Gottschling et al., 2001). Although several studies have relied on ITS sequences to yield the information necessary to reconstruct the phylogeny, none of the studies focusing on mosses have aligned their sequences based on structural constraints on the molecule. The two Internal Transcribed Spacers (ITS1 and ITS2) may not code for RNA molecules but play a role in the maturation of the rRNA subunits, and both spacers have a primary sequence from which a secondary structure can be derived (e.g., Goertzen et al., 2003), and this structure seems highly conserved among plants (Coleman, 2003). Similarly, the alignment of the *trn*L intron, which is widely used in moss phylogenetics, should also be didacted by models of the secondary structure (e.g., Borsch et al., 2003; Stech et al., 2003a). Quandt et al. (2003) described a dyad symmetrical element in the *psb*N-*psb*H spacer (cpDNA) and small inversions that occurred in the loop of the hair-pin structure. If no effort had been made to test for, and hence predict, a secondary structural model, then the inversions would have remained unnoticed, and homology assumptions flawed. Indeed, Quandt et al. (2003) demonstrated that these

Figure 1. Summary of current state of phylogenetic resolution for families of mosses, based on inferences from DNA sequence data (see text review). Taxa of ambiguous affinities are retained in a conservative position.

mutations are highly homoplasic even among genera of pleurocarpous mosses, and also suggested that the inclusion of the loop in the analyses significantly decreased the robustness of the results. It, thus, appears imperative to explore secondary structure models for primary sequence data in order to extract a robust and accurate signal.

Taking into account the folding of the molecule allows also the detection of any compensatory changes that occur on complementary portions of the sequence (Álvarez & Wendel, 2003; Coleman, 2003; Quandt et al., 2003). In the case of the *trn*L intron, Quandt et al. (2004) argue that these base changes contribute significant phylogenetic signal only when deep nodes of the evolutionary tree of land plants are considered, but that they may have little effect if apical clades such as the pleurocarpous mosses are considered. However, considering that pleurocarpous mosses are of fairly recent origin and have undergone a rapid diversification (Shaw et al., 2003b), it is not clear if Quandt et al.'s observation will apply to other groups of mosses.

During the last ten years, bryologists have overcome the difficulties of extracting, amplifying, and sequencing DNA from fresh as well as older herbarium material to test phylogenetic hypotheses. The data derived from DNA sequencing have permitted new insights into moss phylogeny. Inferences from DNA sequences may corroborate the hypothesis drawn from morphological characters (e.g., monophyly of the Splachnaceae: Goffinet et al., 2004a), resolve relationships that were left ambiguous based on morphology (e.g., affinities of the Encalyptaceae: Goffinet & Cox, 2000), or lead to a reevaluation of homology assumptions of morphological characters in cases of severe conflict (e.g., circumscription of the Bryaceae and Mniaceae: Cox & Hedderson, 1999; 2003). Morphological characters define the null hypotheses that are being tested using sequence data, and they remain central to our interpretations of phylogenetic hypotheses. However, as is evident from the present review, most morphological characters traditionally used to defined taxonomic entities of bryophytes are poor phylogenetic indicators, although others may be revealed to hold the signal that marks the origin of a

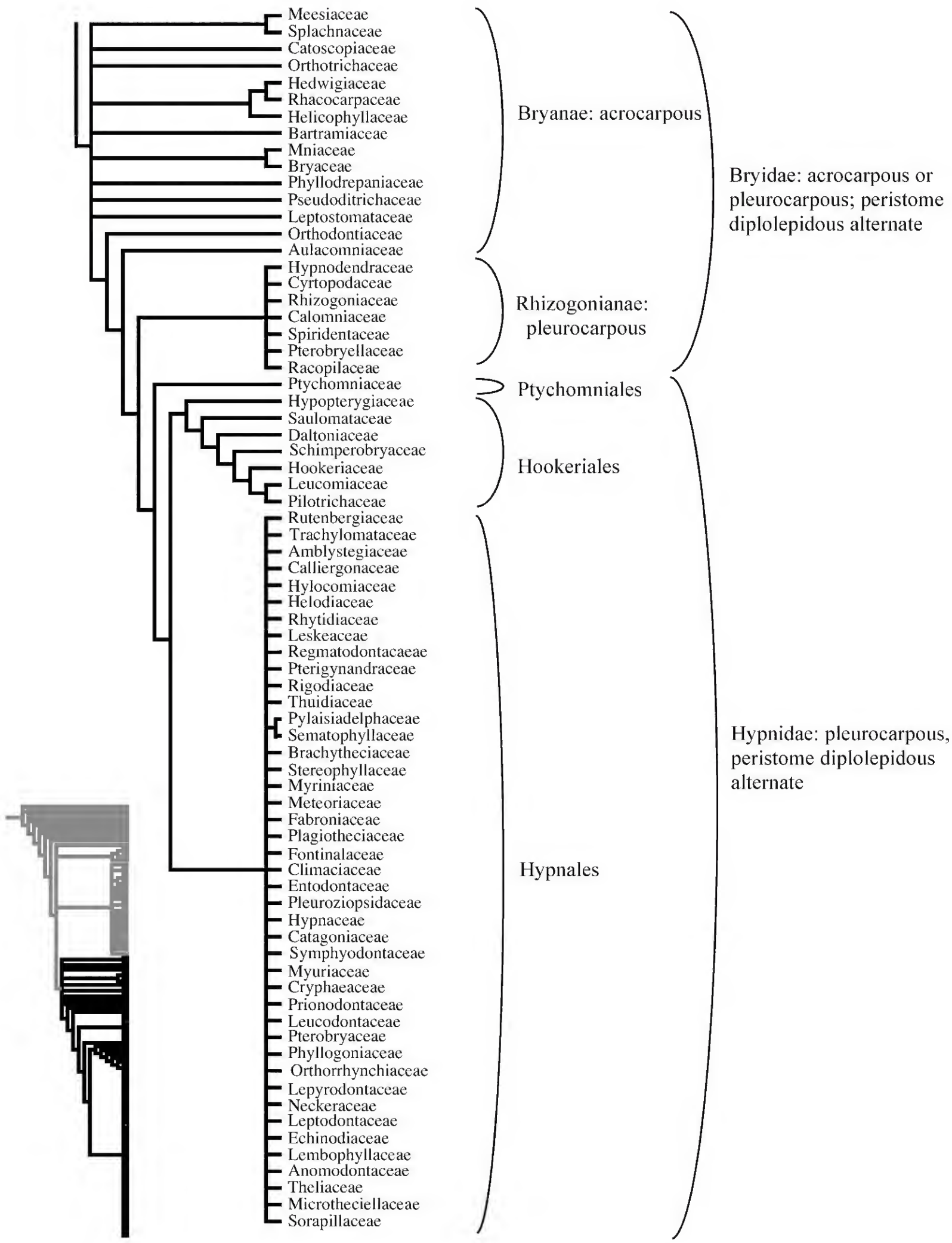

Figure 1. Continued.

clade. As bryologists move into the 21st century, all sources of data that allow for a better resolution of phylogenetic relationships are needed, and molecular sequences offer the best hope for understanding not only familial circumscriptions and phylogeny, but ultimately also the evolution of morphological characters. It is, indeed, the history of morphological transformations that define taxa or of the distribution ranges of the species that justify the investment into phylogenetic approaches. A re-

vival of critical morphological and anatomical studies is, however, imperative if major clades of mosses are to be diagnosed by characters other than their genomes.

Classification of the Bryophyta

From the preceding review it is clear that inferences from molecular data contribute significantly to our understanding of the evolutionary relationships among mosses. Molecules have been essential to resolve the affinities of taxa with reduced morphologies (e.g., *Ephemerum*, *Oedipodium*), and to reveal that similarities in morphological characters may result from convergence rather than shared ancestry (e.g., Leucodontales). As a consequence, several taxa appear to be polyphyletic (e.g., *Bryum*, Bryaceae). Monophyly of the ingroup is an essential assumption to the reconstruction of the phylogeny and its interpretation in terms of character evolution. Monophyly may be violated if taxa of distant relationship are included in the ingroup and if others, currently accommodated in distinct lineages, are omitted from the ingroup. To test whether any taxa are missing from the ingroup is an impossible task, as exemplars of all outgroup taxa must be sampled. However, testing the shared ancestry of taxa assumed to be members of the ingroup is more readily done, especially as the number of available sequences to include in the outgroup is growing. Goffinet et al. (2001) argued that the *rps*4 gene of all ingroup taxa (or their exemplars) should be sequenced and inserted in the large matrix, simply to provide a preliminary test of the monophyly of the group. Although it is not a robust test, the *rps*4 data set is currently the largest available one covering a broad systematic spectrum of mosses. The affinities of monospecific genera or monogeneric families in the Bryophyta could be easily addressed using this broad character sample.

Novel hypotheses drawn from the analyses of molecular data (see Fig. 1 for summary) are integrated here (Appendix 1) into the classification of mosses recently revised by Buck and Goffinet (2000). These amendments to the circumscription of taxa are complemented by several studies based on patterns emerging from the variation of morphological characters. The classification proposed here represents only our best interpretation of the available evidence. In cases of incongruence, we have favored morphology when alternative hypotheses seem poorly supported by molecular data. Many studies have revealed the polyphyly of large genera, but failed to offer new systematic concepts; hence, these moss taxa are retained here in their traditional sense.

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APPENDIX 1. Classification of the mosses.

BRYOPHYTA Schimp.:
SUPERCLASS I
CLASS TAKAKIOPSIDA (Crand.-Stotl.) Goffinet & W. R. Buck, comb. et stat. nov. Takakiophyta Crandall-Stotler, J. Bryol. 14: 17. 1986.
ORDER TAKAKIALES S. Hatt. & H. Inoue
Takakiaceae S. Hatt. & Inoue Type: *Takakia* S. Hatt. & Inoue
SUPERCLASS II
CLASS SPHAGNOPSIDA (Engl.) Ochyra
ORDER SPHAGNALES Limpr.
Sphagnaceae Dumort. Type: *Sphagnum* L.
ORDER AMBUCHANANIALES Seppelt & H. A. Crum
Ambuchananiaceae Seppelt & H. A. Crum Type: *Ambuchanania* Seppelt & H. A. Crum
SUPERCLASS III
CLASS ANDREAEOPSIDA (Limpr.) Rothm.
ORDER ANDREAEALES Limpr.
Andreaeaceae Dumort. Type: *Andreaea* Hedw.
Acroschisma Lindl., *Andreaea* Hedw.
SUPERCLASS IV
CLASS ANDREAEOBRYOPSIDA (B. M. Murray) Goffinet & W. R. Buck, comb. et stat. nov. Andreaeobryales B. M. Murray, Beih. Nova Hedwigia 90: 299. 1988.
ORDER ANDREAEOBRYALES B. M. Murray
Andreaeobryaceae Steere & B. M. Murray Type: *Andreaeobryum* Steere & B. M. Murray
SUPERCLASS V
CLASS OEDIPODIOPSIDA (Schimp.) Goffinet & W. R. Buck, comb. et stat. nov. Oedipodiaceae Schimp., Syn. Musc. Eur. ed. 2, XCVIII, 354. 1876.
ORDER OEDIPODIALES (Schimp.) Goffinet & W. R. Buck, comb. et stat. nov. Oedipodiaceae Schimp., Syn. Musc. Eur. ed. 2, XCVIII, 354. 1876.
Oedipodiaceae Schimp. Type: *Oedipodium* Schwägr.
CLASS POLYTRICHOPSIDA Ochyra, Zarnowiec & Bednarek-Ochyra
ORDER POLYTRICHALES M. Fleisch.
Polytrichaceae Schwägr. Type: *Polytrichum* Hedw.
Alophozia Card., *Atrichopsis* Card., *Atrichum* P. Beauv., *Bartramiopsis* Kindb., *Dawsonia* R. Br., *Dendroligotrichum* (Müll. Hal.) Broth., *Hebantia* G. L. S. Merr., *Itatiella* G. L. Sm., *Lyellia* R. Br., *Meiotrichum* (G. L. Sm.) G. L. S. Merr., *Notoligotrichum* G. L. Sm., *Oligotrichum* Lam. & DC., *Plagioracelopus* G. L. S. Merr., *Pogonatum* P. Beauv., *Polytrichadelphus* (Müll. Hal.) Mitt., *Polytrichastrum* G. L. Sm., *Polytrichum* Hedw., *Pseudatrichum* Reimers, *Pseudoracelopus* Broth., *Psilopilum* Brid., *Racelopodopsis* Thér., *Racelopus* Dozy & Molk., *Steereobryon* G. L. Sm.
CLASS TETRAPHIDOPSIDA (M. Fleisch.) Goffinet & W. R. Buck, comb. et stat. nov. Bryales subordo Tetraphidiineae M. Fleisch., Musci Fl. Buitenzorg 3: xvi. 1908.
ORDER TETRAPHIDALES M. Fleisch.
Tetraphidaceae Schimp. Type: *Tetraphis* Hedw.
Tetraphis Hedw., *Tetrodontium* Schwägr.
CLASS BRYOPSIDA (Limpr.) Rothm.
SUBCLASS BUXBAUMIIDAE (M. Fleisch.) Ochyra
ORDER BUXBAUMIALES M. Fleisch.
Buxbaumiaceae Schimp. Type: *Buxbaumia* Hedw.
SUBCLASS DIPHYSCIIDAE (M. Fleisch.) Ochyra
ORDER DIPHYSCIALES M. Fleisch.
Diphysciaceae M. Fleisch. Type: *Diphyscium* D. Mohr
SUBCLASS FUNARIIDAE (W. Frey) Ochyra
ORDER TIMMIALES (M. Fleisch.) Ochyra
Timmiaceae Schimp. Type: *Timmia* Hedw.
ORDER ENCALYPTALES Dixon
Encalyptaceae Schimp. Type: *Encalypta* Hedw.
Bryobartramia Sainsb., *Bryobrittonia* R. S. Williams, *Encalypta* Hedw.
ORDER FUNARIALES M. Fleisch.
Funariaceae Schwägr. Type: *Funaria* Hedw.

Appendix 1. Continued.

Aphanorhegma Sull., *Brachymeniopsis* Broth., *Bryobeckettia* Fife, *Corynotheca* Ochyra, *Cygnicollum* Fife & Magill, *Entosthodon* Schwägr., *Funaria* Hedw., *Funariella* Sérgio, ×*Funariophyscomitrella* Wettst., *Loiseaubryum* Bizot, *Nanomitriella* E. B. Bartram, *Physcomitrella* Bruch & Schimp., *Physcomitrellopsis* Broth. & Wager, *Physcomitrium* (Brid.) Brid., *Pyramidula* Brid.

Disceliaceae Schimp. Type: *Discelium* Brid.

Gigaspermaceae Lindb. Type: *Gigaspermum* Lindb.

Chamaeobryum Thér. & Dixon, *Costesia* Thér., *Gigaspermum* Lindb., *Lorentziella* Müll. Hal., *Oedipodiella* Dixon

Subclas Dicranidae (W. Frey) Ochyra

Order Scouleriales (S. P. Churchill) Goffinet & W. R. Buck, comb. et stat. nov. Scouleriaceae S. P. Churchill in Funk & D. R. Brooks, Advances Cladistics 143. 1981.

Scouleriaceae S. P. Churchill Type: *Scouleria* Hook.

Scouleria Hook., *Tridontium* Hook. f.

Drummondiaceae (Vitt) Goffinet Type: *Drummondia* Hook.

Order Bryoxiphiales H. A. Crum & L. E. Anderson

Bryoxiphiaceae Besch. Type: *Bryoxiphium* Mitt.

Order Grimmiales M. Fleisch.

Grimmiaceae Arn. Type: *Grimmia* Hedw.

Aligrimmia R. S. Williams, *Bucklandiella* Roiv., *Codriophorus* P. Beauv., *Coscinodon* Spreng., *Coscinodontella* R. S. Williams, *Dryptodon* Brid., *Grimmia* Hedw., *Guembelia* Hampe, *Hydrogrimmia* (I. Hagen) Loeske, *Indusiella* Broth. & Müll. Hal., *Jaffueliobryum* Thér., *Leucoperichaetium* Magill, *Niphotrichum* (Bednarek-Ochyra) Bednarek-Ochyra & Ochyra, *Orthogrimmia* (Schimp.) Ochyra & Zarnowiec, *Racomitrium* Brid., *Schistidium* Bruch & Schimp., *Streptocolea* (I. Hagen) Ochyra & Zarnowiec

Ptychomitriaceae Schimp. Type: *Ptychomitrium* Fürnr.

Campylostelium Bruch & Schimp., *Ptychomitriopsis* Dixon, *Ptychomitrium* Fürnr.

Seligeriaceae Schimp. Type: *Seligeria* Bruch & Schimp.

Blindia Bruch & Schimp., *Brachydontium* Fürnr., *Hymenolomopsis* Thér., *Seligeria* Bruch & Schimp., *Trochobryum* Breidl. & Beck

Order Archidiales Limpr.

Archidiaceae Schimp. Type: *Archidium* Brid.

Order Dicranales H. Philib. ex M. Fleisch.

Fissidentaceae Schimp. Type: *Fissidens* Hedw.

Eustichiaceae Broth. Type: *Eustichia* (Brid.) Brid.

Ditrichaceae Limpr. Type: *Ditrichum* Hampe

Astomiopsis Müll. Hal., *Austrophilibertiella* Ochyra, *Bryomanginia* Thér., *Ceratodon* Brid., *Cheilothela* Broth., *Chrysoblastella* R. S. Williams, *Cladastomum* Müll. Hal., *Cleistocarpidium* Ochyra & Bednarek-Ochyra, *Crumuscus* W. R. Buck & Snider, *Cygniella* H. A. Crum, *Distichium* Bruch & Schimp., *Ditrichopsis* Broth., *Ditrichum* Hampe, *Eccremidium* Hook. f. & Wilson, *Garckea* Müll. Hal., *Kleioweisopsis* Dixon, ×*Pleuriditrichum* A. L. Andrews & F. J. Herm., *Pleuridium* Rabenh., *Rhamphidium* Mitt., *Saelania* Lindb., *Skottsbergia* Cardot, *Strombulidens* W. R. Buck, *Trichodon* Schimp., *Tristichium* Müll. Hal., *Wilsoniella* Müll. Hal.

Bruchiaceae Schimp. Type: *Bruchia* Schwägr.

Bruchia Schwägr., *Cladophascum* Sim, *Eobruchia* W. R. Buck, *Pringleella* Cardot, *Trematodon* Michx.

Rhabdoweisiaceae Limpr. Type: *Rhabdoweisia* Bruch & Schimp.

Amphidium Schimp., *Arctoa* Bruch & Schimp., *Cynodontium* Schimp., *Dichodontium* Schimp., *Dicranoweisia* Milde, *Glyphomitrium* Brid., *Holodontium* (Mitt.) Broth., *Hymenoloma* Dusén, *Kiaeria* I. Hagen, *Oncophorus* (Brid.) Brid., *Oreas* Brid., *Oreoweisia* (Bruch & Schimp.) De Not., *Pseudohyophila* Hilp., *Rhabdoweisia* Bruch & Schimp., *Symblepharis* Mont., *Verrucidens* Cardot

Rhachitheciaceae H. Rob. Type: *Rhachithecium* Le Jolis

Hypnodontopsis Z. Iwats. & Nog., *Jonesiobryum* B. H. Allen & Pursell, *Rhachitheciopsis* P. de la Varde, *Rhachithecium* Le Jolis, *Tisserantiella* P. de la Varde, *Uleastrum* W. R. Buck, *Zanderia* Goffinet

Erpodiaceae Broth. Type: *Erpodium* (Brid.) Brid.

Aulacopilum Wilson, *Erpodium* (Brid.) Brid., *Solmsiella* Müll. Hal., *Venturiella* Müll. Hal., *Wildia* Müll. Hal. & Broth.

Mitteniaceae Broth. Type: *Mittenia* Lindb.

Schistostegaceae Schimp. Type: *Schistotega* D. Mohr

Viridivelleraceae I. G. Stone Type: *Viridivellus* I. G. Stone

Appendix 1. Continued.

Dicranaceae Schimp. Type: *Dicranum* Hedw.

Anisothecium Mitt., *Aongstroemia* Bruch & Schimp., *Aongstroemiopsis* M. Fleisch., *Braunfelsia* Paris, *Brotherobryum* M. Fleisch., *Bryotestua* Thér. & P. de la Varde, *Camptodontium* Dusén, *Campylopodium* (Müll. Hal.) Besch., *Chorisodontium* (Mitt.) Broth., *Cnestrum* I. Hagen, *Cryptodicranum* E. B. Bartram, *Dicnemon* Schwägr., *Dicranella* (Müll. Hal.) Schimp., *Dicranoloma* (Renauld) Renauld, *Dicranum* Hedw., *Diobelonella* Ochyra, *Eucamptodon* Mont., *Eucamptodontopsis* Broth., *Holomitriopsis* H. Rob., *Holomitrium* Brid., *Hygrodicranum* Cardot, *Leptotrichella* (Müll. Hal.) Lindb., *Leucoloma* Brid., *Macrodictyum* (Broth.) E. H. Hegew., *Mesotus* Mitt., *Mitrobryum* H. Rob., *Muscoherzogia* Ochyra, *Orthodicranum* (Bruch & Schimp.) Loeske, *Paraleucobryum* (Limpr.) Loeske, *Parisia* Broth., *Platyneuron* (Cardot) Broth., *Pocsiella* Bizot, *Polymerodon* Herzog, *Pseudephemerum* (Lindb.) I. Hagen, *Pseudochorisodontium* (Broth.) C. H. Gao, Vitt, X. H. Fu & T. Cao, *Schliephackea* Müll. Hal., *Sclerodontium* Schwägr., *Sphaerothecium* Hampe, *Steyermarkiella* H. Rob., *Wardia* Harv. & Hook., *Werneriobryum* Herzog

Leucobryaceae Schimp. Type: *Leucobryum* Hampe

Atractylocarpus Mitt., *Brothera* Müll. Hal., *Bryohumbertia* P. de la Varde & Thér., *Campylopodiella* Cardot, *Campylopus* Brid., *Cladopodanthus* Dozy & Molk., *Dicranodontium* Bruch & Schimp., *Leucobryum* Hampe, *Microcampylopus* (Müll. Hal.) Fleisch., *Ochrobryum* Mitt., *Pilopogon* Brid., *Schistomitrium* Dozy & Molk.

Calymperaceae Kindb. Type: *Calymperes* Sw.

Arthrocormus Dozy & Molk., *Calymperes* Sw., *Exodictyon* Cardot, *Exostratum* L. T. Ellis, *Leucophanes* Brid., *Mitthyridium* H. Rob., *Octoblepharum* Hedw., *Syrrhopodon* Schwägr.

Order Pottiales M. Fleisch.

Pottiaceae Schimp. Type: *Pottia* (Reichenbach) Fürnr.

Acaulon Müll. Hal., *Aloinia* Kindb., *Aloinella* Cardot, *Anoectangium* Schwägr., *Aschisma* Lindb., *Barbula* Hedw., *Bellibarbula* P. C. Chen, *Bryoceuthospora* H. A. Crum & L. E. Anderson, *Bryoerythrophyllum* P. C. Chen, *Calymperastrum* I. G. Stone, *Calyptopogon* (Mitt.) Broth., *Chenia* R. H. Zander, *Chionoloma* Dixon, *Crossidium* Jur., *Crumia* W. B. Schofield, *Dialytrichia* (Schimp.) Limpr., *Didymodon* Hedw., *Dolotortula* R. H. Zander, *Ephemerum* Schimp., *Erythrophyllastrum* R. H. Zander, *Erythrophyllopsis* Broth., *Eucladium* Bruch & Schimp., *Ganguleea* R. H. Zander, *Gertrudiella* Broth., *Globulinella* Steere, *Goniomitrium* Hook. f. & Wilson, *Gymnostomiella* M. Fleisch., *Gymnostomum* Nees & Hornsch., *Gyroweisia* Schimp., *Hennediella* Paris, *Hilpertia* R. H. Zander, *Hymenostyliella* E. B. Bartram, *Hymenostylium* Brid., *Hyophila* Brid., *Hyophiladelphus* (Müll. Hal.) R. H. Zander, *Hypodontium* Müll. Hal., *Leptobarbula* Schimp., *Leptodontiella* R. H. Zander & E. H. Hegew., *Leptodontium* (Müll. Hal.) Lindb., *Luisierella* Thér. & P. de la Varde, *Microbryum* Schimp., *Microcrossidium* J. Guerra & M. J. Cano, *Micromitrium* Austin, *Mironia* R. H. Zander, *Molendoa* Lindb., *Nanomitriopsis* Cardot, *Neophoenix* R. H. Zander & During, *Pachyneuropsis* H. Mill., *Phascopsis* I. G. Stone, *Plaubelia* Brid., *Pleurochaete* Lindb., *Pottiopsis* Blockeel & A. J. E. Sm., *Pseudocrossidium* R. S. Williams, *Pseudosymblepharis* Broth., *Pterygoneurum* Jur., *Quaesticula* R. H. Zander, *Reimersia* P. C. Chen, *Rhexophyllum* Herzog, *Sagenotortula* R. H. Zander, *Saitobryum* R. H. Zander, *Sarconeurum* Bryhn, *Scopelophila* (Mitt.) Lindb., *Splachnobryum* Müll. Hal., *Stegonia* Venturi, *Stonea* R. H. Zander, *Streptocalypta* Müll. Hal., *Streptopogon* Mitt., *Streptotrichum* Herzog, *Syntrichia* Brid., *Teniolophora* W. D. Reese, *Tetracoscinodon* R. Br., *Tetrapterum* A. Jaeger, *Timmiella* (De Not.) Schimp., *Tortella* (Lindb.) Limpr., *Tortula* Hedw., *Trachycarpidium* Broth., *Trachyodontium* Steere, *Trichostomum* Bruch, *Triquetrella* Müll. Hal., *Tuerckheimia* Broth., *Uleobryum* Broth., *Weisiopsis* Broth., *Weissia* Hedw., *Weissiodicranum* W. D. Reese, *Willia* Müll. Hal.

Pleurophascaceae Broth. Type: *Pleurophascum* Lindb.

Serpotortellaceae W. D. Reese & R. H. Zander Type: *Serpotortella* Dixon

Cinclidotaceae K. Saito Type: *Cinclidotus* P. Beauv.

Subclass Bryidae Engl.

Superorder Bryanae (Engl.) Goffinet & W. R. Buck, comb. et stat. nov. Bryidae Engl. in Engl. & K. Prantl, Nat. Pflanzenfam. 1(3): 2. 1893.

Order Splachnales (M. Fleisch.) Ochyra

Splachnaceae Grev. & Arn. Type: *Splachnum* Hedw.

Aplodon R. Br., *Moseniella* Broth., *Splachnum* Hedw., *Tayloria* Hook., *Tetraplodon* Bruch & Schimp., *Voitia* Hornsch.

Meesiaceae Schimp. Type: *Meesia* Hedw.

Amblyodon P. Beauv., *Leptobryum* (Bruch & Schimp.) Wilson, *Meesia* Hedw., *Neomeesia* Deguchi, *Paludella* Brid.

Appendix 1. Continued.

Order Orthotrichales Dixon
Orthotrichaceae Arn. Type: *Orthotrichum* Hedw.
Cardotiella Vitt, *Ceuthotheca* Lewinsky, *Codonoblepharon* Schwägr., *Desmotheca* Lindb., *Florschuetziella* Vitt, *Groutiella* Steere, *Leiomitrium* Mitt., *Leratia* Broth. & Paris, *Macrocoma* (Müll. Hal.) Grout, *Macromitrium* Brid., *Matteria* Goffinet, *Orthotrichum* Hedw., *Pentastichella* Müll. Hal., *Pleurorthotrichum* Broth., *Schlotheimia* Brid., *Sehnemobryum* Lewinsky-Haapasaari & Hedenäs, *Stoneobryum* D. H. Norris & H. Rob., *Ulota* D. Mohr, *Zygodon* Hook. & Taylor
Order Hedwigiales Ochyra
Hedwigiaceae Schimp. Type: *Hedwigia* P. Beauv.
Braunia Bruch & Schimp., *Bryowijkia* Nog., *Hedwigia* P. Beauv., *Hedwigidium* Bruch & Schimp., *Pseudobraunia* (Lesq. & James) Broth.
Helicophyllaceae Broth. Type: *Helicophyllum* Brid.
Rhacocarpaceae Kindb. Type: *Rhacocarpus* Lindb.
Pararhacocarpus Frahm, *Rhacocarpus* Lindb.
Order Bryales Limpr.
Catoscopiaceae Broth. Type: *Catoscopium* Brid.
Bartramiaceae Schwägr. Type: *Bartramia* Hedw.
Anacolia Schimp., *Bartramia* Hedw., *Breutelia* (Bruch & Schimp.) Schimp., *Conostomum* Sw., *Fleischerobryum* Loeske, *Flowersia* D. G. Griffin & W. R. Buck, *Leiomela* (Mitt.) Broth., *Neosharpiella* H. Rob. & Delgad., *Philonotis* Brid., *Plagiopus* Brid.
Bryaceae Schwägr. Type: *Bryum* Hedw.
Acidodontium Schwägr., *Anomobryum* Schimp., *Brachymenium* Schwägr., *Bryum* Hedw., *Leptostomopsis* (Müll. Hal.) J. R. Spence & H. P. Ramsay, *Mniobryoides* Hörmann, *Osculatia* De Not., *Perssonia* Bizot, *Plagiobryum* Lindb., *Rhodobryum* (Schimp.) Limpr., *Roellia* Kindb., *Rosulabryum* J. R. Spence
Phyllodrepaniaceae Crosby Type: *Phyllodrepanium* Crosby
Mniomalia Müll. Hal., *Phyllodrepanium* Crosby
Pseudoditrichaceae Steere & Z. Iwats. Type: *Pseudoditrichum* Steere & Z. Iwats.
Mniaceae Schwägr. Type: *Mnium* Hedw.
Cinclidium Sw., *Cyrtomnium* Holmen, *Epipterygium* Lindb., *Leucolepis* Lindb., *Mielichhoferia* Nees & Hornsch., *Mnium* Hedw., *Ochiobryum* J. R. Spence & H. P. Ramsay, *Orthomnion* Wilson, *Plagiomnium* T. J. Kop., *Pohlia* Hedw., *Pseudobryum* (Kindb.) T. J. Kop., *Pseudopohlia* R. S. Williams, *Rhizomnium* (Broth.) T. J. Kop., *Schizymenium* Harv., *Synthetodontium* Cardot, *Trachycystis* T. J. Kop.
Leptostomataceae Schwägr. Type: *Leptostomum* R. Br.
Aulacomniaceae Schimp. Type: *Aulacomnium* Schwägr.
Orthodontiaceae (Broth.) Goffinet Type: *Orthodontium* Wilson
Orthodontium Wilson, *Orthodontopsis* Ignatov & B. C. Tan
Superorder Rhizogonianae (M. Fleisch.) Goffinet & W. R. Buck, comb. et stat. nov. Bryales subordo Rhizogoniineae M. Fleisch., Musci Fl. Buitenzorg 4: xvi. 1923.
Order Rhizogoniales (M. Fleisch.) Goffinet & W. R. Buck, comb. et stat. nov. Bryales subordo Rhizogoniineae M. Fleisch., Musci Fl. Buitenzorg 4: xvi. 1923.
Rhizogoniaceae Broth. Type: *Rhizogonium* Brid.
Cryptopodium Brid., *Goniobryum* Lindb., *Hymenodon* Hook. f. & Wilson, *Hymenodontopsis* Herzog, *Leptotheca* Schwägr., *Mesochaete* Lindb., *Pyrrhobryum* Mitt., *Rhizogonium* Brid.
Calomniaceae Kindb. Type: *Calomnion* Hook. f. & Wilson
Hypnodendraceae Broth. Type: *Hypnodendron* (Müll. Hal.) Mitt.
Cyrtopodaceae M. Fleisch. Type: *Cyrtopus* (Brid.) Hook. f.
Bescherellia Duby, *Cyrtopodendron* M. Fleisch., *Cyrtopus* (Brid.) Hook. f.
Spiridentaceae Kindb. Type: *Spiridens* Nees
Franciella Thér., *Spiridens* Nees
Pterobryellaceae (Broth.) W. R. Buck & Vitt Type: *Pterobryella* (Müll. Hal.) A. Jaeger
Racopilaceae Kindb. Type: *Racopilum* P. Beauv.
Powellia Mitt., *Racopilum* P. Beauv.
Subclass Hypnidae W. R. Buck, Goffinet & A. J. Shaw
Superorder Ptychomnianae W. R. Buck, C. J. Cox, A. J. Shaw & Goffinet
Order Ptychomniales W. R. Buck, C. J. Cox, A. J. Shaw & Goffinet
Ptychomniaceae M. Fleisch. Type: *Ptychonion* (Hook. f. & Wilson) Mitt.

Appendix 1. Continued.

Cladomnion Hook. f. & Wilson, *Cladomniopsis* M. Fleisch., *Dichelodontium* Broth., *Endotrichellopsis* During, *Euptychium* Schimp., *Garovaglia* Endl., *Glyphothecium* Hampe, *Hampeella* Müll. Hal., *Ptychomniella* (Broth.) W. R. Buck, C. J. Cox, A. J. Shaw & Goffinet, *Ptychomnion* (Hook. f. & Wilson) Mitt., *Tetraphidopsis* Broth. & Dixon

Superorder Hypnanae W. R. Buck, C. J. Cox, A. J. Shaw & Goffinet

Order Hookeriales M. Fleisch.

Hypopterygiaceae Mitt. Type: *Hypopterygium* Brid.

Arbusculohypopterygium Stech, T. Pfeiffer & W. Frey, *Canalohypopterygium* W. Frey & Schaepe, *Catharomnion* Hook. f. & Wilson, *Cyathophorella* (Broth.) M. Fleisch., *Cyathophorum* P. Beauv., *Dendrocyathophorum* Dixon, *Dendrohypopterygium* Kruijer, *Hypopterygium* Brid., *Lopidium* Hook. f. & Wilson

Saulomataceae W. R. Buck, C. J. Cox, A. J. Shaw & Goffinet Type: *Sauloma* (Hook. f. & Wils.) Mitt.

Ancistrodes Hampe, *Sauloma* (Hook. f. & Wilson) Mitt., *Vesiculariopsis* Broth.

Daltoniaceae Schimp. Type: *Daltonia* Hook. & Taylor

Achrophyllum Vitt & Crosby, *Adelothecium* Mitt., *Benitotania* H. Akiy., Yamaguchi & Suleiman, *Bryobrothera* Thér., *Calyptrochaeta* Desv., *Crosbya* Vitt, *Beeveria* Fife, *Daltonia* Hook. & Taylor, *Distichophyllidium* M. Fleisch., *Distichophyllum* Dozy & Molk., *Ephemeropsis* K. I. Goebel, *Leskeodon* Broth., *Leskeodontopsis* Zanten, *Metadistichophyllum* Nog. & Z. Iwats.

Schimperobryaceae W. R. Buck, C. J. Cox, A. J. Shaw & Goffinet Type: *Schimperobryum* Margad.

Hookeriaceae Schimp. Type: *Hookeria* J. E. Sm.

Crossomitrium Müll. Hal., *Hookeria* J. E. Sm.

Leucomiaceae Broth. Type: *Leucomium* Mitt.

Leucomium Mitt., *Rhynchostegiopsis* Müll. Hal., *Tetrastichium* (Mitt.) Cardot

Pilotrichaceae Kindb. Type: *Pilotrichum* P. Beauv.

Actinodontium Schwägr., *Amblytropis* (Mitt.) Broth., *Brymela* Crosby & B. H. Allen, *Callicostella* (Müll. Hal.) Mitt., *Callicostellopsis* Broth., *Cyclodictyon* Mitt., *Diploneuron* E. B. Bartram, *Helicoblepharum* (Mitt.) Broth., *Hemiragis* (Brid.) Besch., *Hookeriopsis* (Besch.) A. Jaeger, *Hypnella* (Müll. Hal.) A. Jaeger, *Lepidopilidium* (Müll. Hal.) Broth., *Lepidopilum* (Brid.) Brid., *Neohypnella* E. B. Bartram, *Philophyllum* Müll. Hal., *Pilotrichidium* Besch., *Pilotrichum* P. Beauv., *Stenodesmus* (Mitt.) A. Jaeger, *Stenodictyon* (Mitt.) A. Jaeger, *Thamniopsis* (Mitt.) M. Fleisch., *Trachyxiphium* W. R. Buck

Order Hypnales (M. Fleisch.) W. R. Buck & Vitt

Rutenbergiaceae (Broth.) M. Fleisch. Type: *Rutenbergia* Besch.

Neorutenbergia Bizot & Pócs, *Pseudocryphaea* Broth., *Rutenbergia* Besch.

Trachylomataceae (M. Fleisch.) W. R. Buck & Vitt Type: *Trachyloma* Brid.

Braithwaitea Lindb., *Trachyloma* Brid.

Fontinalaceae Schimp. Type: *Fontinalis* Hedw.

Brachelyma Cardot, *Dichelyma* Myrin, *Fontinalis* Hedw.

Climaciaceae Kindb.: *Climacium* F. Weber & D. Mohr

Pleuroziopsidaceae Ireland Type: *Pleuroziopsis* E. Britton

Amblystegiaceae G. Roth Type: *Amblystegium* Schimp.

Amblystegium Schimp., *Anacamptodon* Brid., *Bryostreimannia* Ochyra, *Campyliadelphus* (Kindb.) R. S. Chopra, *Campylium* (Sull.) Mitt., *Conardia* H. Rob., *Cratoneuron* (Sull.) Spruce, *Cratoneuropsis* (Broth.) M. Fleisch., *Drepanocladus* (Müll. Hal.) G. Roth, *Gradsteinia* Ochyra, *Hygroamblystegium* Loeske, *Hygrohypnum* Lindb., *Hypnobartlettia* Ochyra, *Koponenia* Ochyra, *Leptodictyum* (Schimp.) Warnst., *Limbella* (Müll. Hal.) Müll. Hal., *Palustriella* Ochyra, *Pictus* C. C. Towns., *Pseudo-calliergon* (Limpr.) Loeske, *Sanionia* Loeske, *Sasaokaea* Broth., *Sciaromiella* Ochyra, *Sciaromiopsis* Broth., *Scorpidium* (Schimp.) Limpr., *Sinocalliergon* Sakurai, *Serpoleskea* (Limpr.) Loeske, *Vittia* Ochyra

Calliergonaceae (Kanda) Vanderpoorten, Hedenäs, C. J. Cox & A. J. Shaw Type: *Calliergon* (Sull.) Kindb.

Calliergon (Sull.) Kindb., *Hamatocaulis* Hedenäs, *Loeskypnum* H. K. G. Paul, *Straminergon* Hedenäs, *Warnstorfia* Loeske

Helodiaceae (M. Fleisch.) Ochyra Type: *Helodium* Warnst.

Actinothuidium (Besch.) Broth., *Bryochenia* C. H. Gao & K. C. Chang, *Helodium* Warnst.

Rigodiaceae H. A. Crum Type: *Rigodium* Schwägr.

Leskeaceae Schimp. Type: *Leskea* Hedw.

Claopodium (Lesq. & James) Renauld & Cardot, *Fabronidium* Müll. Hal., *Haplocladium* (Müll. Hal.) Müll. Hal., *Hylocomiopsis* Cardot, *Leptocladium* Broth., *Leptopterigynandrum* Müll. Hal., *Lescuraea* Bruch & Schimp., *Leskea* Hedw., *Leskeadelphus* Herzog, *Leskeella* (Limpr.) Loeske, *Lindbergia* Kindb., *Mamillariella* Laz., *Miyabea* Broth., *Orthoamblystegium* Dixon & Sakurai, *Platylomella* A. L. Andrews, *Pseudoleskea* Bruch & Schimp., *Pseudoleskeella* Kindb., *Pseudoleskeopsis* Broth., *Ptychodium* Schimp., *Rigodiadelphus* Dixon, *Rozea* Besch., *Schwetschkea* Müll. Hal.

Appendix 1. Continued.

Thuidiaceae Schimp. Type: *Thuidium* Bruch & Schimp.
Abietinella Müll. Hal., *Boulaya* Cardot, *Cyrto-hypnum* (Hampe) Hampe & Lorentz, *Fauriella* Besch., *Pelekium* Mitt., *Rauiella* Reimers, *Thuidiopsis* (Broth.) M. Fleisch., *Thuidium* Bruch & Schimp.

Regmatodontaceae Broth. Type: *Regmatodon* Brid.

Stereophyllaceae (M. Fleisch.) W. R. Buck & Ireland Type: *Stereophyllum* Mitt.
Catagoniopsis Broth., *Entodontopsis* Broth., *Eulacophyllum* W. R. Buck & Ireland, *Juratzkaea* Lorentz, *Pilosium* (Müll. Hal.) M. Fleisch., *Sciuroleskea* Broth., *Stenocarpidium* Müll. Hal., *Stereophyllum* Mitt.

Brachytheciaceae G. Roth Type: *Brachythecium* Schimp.
Aerobryum Dozy & Molk., *Aerolindigia* M. Menzel, *Brachytheciastrum* Ignatov & Huttunen, *Brachythecium* Schimp., *Bryhnia* Kaurin, *Bryoandersonia* H. Rob., *Cirriphyllum* Grout, *Clasmatodon* Hook. f. & Wilson, *Donrichardsia* H. A. Crum & L. E. Anderson, *Eriodon* Mont., *Eurhynchiadelphus* Ignatov & Huttunen, *Eurhynchiastrum* Ignatov & Huttunen, *Eurhynchiella* M. Fleisch., *Eurhynchium* Bruch & Schimp., *Flabellidium* Herzog, *Helicodontium* Schwägr., *Homalotheciella* (Cardot) Broth., *Homalothecium* Schimp., *Juratzkaeella* W. R. Buck, *Kindbergia* Ochyra, *Lindigia* Hampe, *Mandoniella* Herzog, *Meteoridium* (Müll. Hal.) Manuel, *Myuroclada* Besch., *Nobregaea* Hedenäs, *Okamuraea* Broth., *Oxyrrhynchium* (Schimp.) Warnst., *Palamocladium* Müll. Hal., *Plasteurhynchium* Broth., *Platyhypnidium* M. Fleisch., *Pseudopleuropus* Takaki, *Pseudoscleropodium* (Limpr.) M. Fleisch., *Remyella* Müll. Hal., *Rhynchostegiella* (Schimp.) Limpr., *Rhynchostegium* Bruch & Schimp., *Schimperella* Thér., *Sciuro-hypnum* (Hampe) Hampe, *Scleropodium* Bruch & Schimp., *Scorpiurium* Schimp., *Squamidium* (Müll. Hal.) Broth., *Stenocarpidiopsis* M. Fleisch., *Tomentypnum* Loeske, *Zelometeorium* Manuel

Meteoriaceae Kindb. Type: *Meteorium* (Brid.) Dozy & Molk.
Aerobryidium M. Fleisch., *Aerobryopsis* M. Fleisch., *Barbella* M. Fleisch., *Barbellopsis* Broth., *Chrysocladium* M. Fleisch., *Cryphaeophilium* M. Fleisch., *Cryptopapillaria* M. Menzel, *Diaphanodon* Renauld & Cardot, *Duthiella* Renauld, *Floribundaria* M. Fleisch., *Lepyrodontopsis* Broth., *Meteoriopsis* Broth., *Meteorium* (Brid.) Dozy & Molk., *Neodicladiella* W. R. Buck, *Neonoguchia* S. H. Lin, *Pseudospiridentopsis* (Broth.) M. Fleisch., *Pseudotrachypus* P. de la Varde & Thér., *Sinskea* W. R. Buck, *Toloxis* W. R. Buck, *Trachycladiella* (M. Fleisch.) M. Menzel & W. Schultze-Motel, *Trachypodopsis* M. Fleisch., *Trachypus* Reinw. & Hornsch.

Myriniaceae Schimp. Type: *Myrinia* Schimp.
Austinia Müll. Hal., *Macgregorella* E. B. Bartram, *Merrilliobryum* Broth., *Myrinia* Schimp., *Nematocladia* W. R. Buck

Fabroniaceae Schimp. Type: *Fabronia* Raddi
Dimerodontium Mitt., *Fabronia* Raddi, *Ischyrodon* Müll. Hal., *Levierella* Müll. Hal., *Rhizofabronia* (Broth.) M. Fleisch.

Hypnaceae Schimp. Type: *Hypnum* Hedw.
Acritodon H. Rob., *Andoa* Ochyra, *Bardunovia* Ignatov & Ochyra, *Breidleria* Loeske, *Bryocrumia* L. E. Anderson, *Callicladium* H. A. Crum, *Calliergonella* Loeske, *Campylidium* (Kindb.) Ochyra, *Campylophyllum* (Schimp.) M. Fleisch., *Caribaeohypnum* Ando & Higuchi, *Chryso-hypnum* (Hampe) Hampe, *Crepidophyllum* Herzog, *Ctenidiadelphus* M. Fleisch., *Cyathothecium* Dixon, *Ectropotheciella* M. Fleisch., *Ectropotheciopsis* (Broth.) M. Fleisch., *Ectropothecium* Mitt., *Elharveya* H. A. Crum, *Elmeriobryum* Broth., *Entodontella* M. Fleisch., *Eurohypnum* Ando, *Foreauella* Dixon & P. de la Varde, *Gammiella* Broth., *Giraldiella* Müll. Hal., *Gollania* Broth., *Hageniella* Broth., *Herzogiella* Broth., *Homomallium* (Schimp.) Loeske, *Hondaella* Dixon & Sakurai, *Horridohypnum* W. R. Buck, *Hyocomium* Bruch & Schimp., *Hypnum* Hedw., *Irelandia* W. R. Buck, *Isopterygiopsis* Z. Iwats., *Leiodontium* Broth., *Leptoischyrodon* Dixon, *Macrothamniella* M. Fleisch., *Mahua* W. R. Buck, *Microctenidium* M. Fleisch., *Mittenothamnium* Henn., *Nanothecium* Dixon & P. de la Varde, *Ochyraea* Vána, *Orthothecium* Bruch & Schimp., *Phyllodon* Bruch & Schimp., *Plagiotheciopsis* Broth., *Platydictya* Berk., *Platygyriella* Cardot, *Podperaea* Z. Iwats. & Glime, *Pseudohypnella* (M. Fleisch.) Broth., *Pseudotaxiphyllum* Z. Iwats., *Ptilium* De Not., *Pylaisia* Schimp., *Pylaisiopsis* (Broth.) Broth., *Rhacopilopsis* Renauld & Cardot, *Rhizohypnella* M. Fleisch., *Sclerohypnum* Dixon, *Stenotheciopsis* Broth., *Stereodontopsis* R. S. Williams, *Syringothecium* Mitt., *Taxiphyllopsis* Higuchi & Deguchi, *Taxiphyllum* M. Fleisch., *Tripterocladium* (Müll. Hal.) A. Jaeger, *Vesicularia* (Müll. Hal.) Müll. Hal., *Wijkiella* Bizot & Lewinsky

Catagoniaceae W. R. Buck & Ireland Type: *Catagonium* Broth.

Pterigynandraceae Schimp. Type: *Pterigynandrum* Hedw.
Habrodon Schimp., *Heterocladium* Bruch & Schimp., *Iwatsukiella* W. R. Buck & H. A. Crum, *Myurella* Bruch & Schimp., *Pterigynandrum* Hedw., *Trachyphyllum* A. Gepp

Appendix 1. Continued.

Hylocomiaceae (Broth.) M. Fleisch. Type: *Hylocomium* Bruch & Schimp.
Ctenidium (Schimp.) Mitt., *Hylocomiastrum* Broth., *Hylocomium* Bruch & Schimp., *Leptocladiella* M. Fleisch., *Leptohymenium* Schwägr., *Loeskeobryum* Broth., *Macrothamnium* M. Fleisch., *Meteoriella* S. Okamura, *Neodolichomitra* Nog., *Orontobryum* M. Fleisch., *Pleurozium* Mitt., *Puiggariopsis* M. Menzel, *Rhytidiadelphus* (Limpr.) Warnst., *Rhytidiopsis* Broth., *Schofieldiella* W. R. Buck

Rhytidiaceae Broth. Type: *Rhytidium* (Sull.) Kindb.

Symphyodontaceae M. Fleisch. Type: *Symphyodon* Mont.
Chaetomitriopsis M. Fleisch., *Chaetomitrium* Dozy & Molk., *Dimorphocladon* Dixon, *Symphyodon* Mont., *Trachythecium* M. Fleisch., *Unclejackia* Ignatov, T. Kop. & D. Norris

Plagiotheciaceae (Broth.) M. Fleisch. Type: *Plagiothecium* Bruch & Schimp.
Buckiella Ireland, *Plagiothecium* Bruch & Schimp., *Struckia* Müll. Hal.

Entodontaceae Kindb. Type: *Entodon* Müll. Hal.
Entodon Müll. Hal., *Erythrodontium* Hampe, *Mesonodon* Hampe, *Pylaisiobryum* Broth.

Pylaisiadelphaceae fam. nov.
Sematophyllaceis affine, cellulis exothecii non collenchymatosis cellulis alaribus parviore plerumque non inflatis pseudoparaphylliis saepe filamentosis differt. Typus: *Pylaisiadelpha* Cardot, *Revue Bryologique* 39: 57. 1912.
Aptychella (Broth.) Herzog, *Brotherella* M. Fleisch., *Clastobryopsis* M. Fleisch., *Clastobryum* Dozy & Molk., *Heterophyllium* (Schimp.) Kindb., *Isocladiella* Dixon, *Isopterygium* Mitt., *Mastopoma* Cardot, *Platygyrium* Bruch & Schimp., *Pterogonidium* Broth., *Pseudotrismegistia* H. Akiy. & Tsubota, *Pylaisiadelpha* Cardot, *Taxitheliella* Dixon, *Taxithelium* Mitt., *Trismegistia* (Müll. Hal.) Müll. Hal., *Wijkia* H. A. Crum

Sematophyllaceae Broth. Type: *Sematophyllum* Mitt.
Acanthorrhynchium M. Fleisch., *Acroporium* Mitt., *Allionellopsis* Ochyra, *Aptychopsis* (Broth.) M. Fleisch., *Chinostomum* Müll. Hal., *Clastobryella* M. Fleisch., *Clastobryophilum* M. Fleisch., *Colobodontium* Herzog, *Donnellia* Austin, *Hydropogon* Brid., *Hydropogonella* Cardot, *Macrohymenium* Müll. Hal., *Meiotheciella* B. C. Tan, W. B. Schofield & H. P. Ramsay, *Meiothecium* Mitt., *Papillidiopsis* (Broth.) W. R. Buck & B. C. Tan, *Paranapiacabaea* W. R. Buck & Vital, *Potamium* Mitt., *Pterogoniopsis* Müll. Hal., *Piloecium* (Müll. Hal.) Broth., *Radulina* W. R. Buck & B. C. Tan, *Rhaphidostichum* M. Fleisch., *Schraderella* Müll. Hal., *Schroeterella* Herzog, *Sematophyllum* Mitt., *Timotimius* W. R. Buck, *Trichosteleum* Mitt., *Trolliella* Herzog, *Warburgiella* Müll. Hal.

Cryphaeaceae Schimp. Type: *Cryphaea* D. Mohr
Cryphaea D. Mohr, *Cryphidium* (Mitt.) A. Jaeger, *Cyptodon* (Broth.) M. Fleisch., *Cyptodontopsis* Dixon, *Dendroalsia* E. Britton, *Dendrocryphaea* Broth., *Dendropogonella* E. Britton, *Pilotrichopsis* Besch., *Schoenobryum* Dozy & Molk., *Sphaerotheciella* M. Fleisch.

Prionodontaceae Broth. Type: *Prionodon* Müll. Hal.

Leucodontaceae Schimp. Type: *Leucodon* Schwägr.
Antitrichia Brid., *Dozya* Sande Lac., *Eoleucodon* H. A. Mill. & H. Whittier, *Felipponea* Broth., *Leucodon* Schwägr., *Pterogonium* Sw., *Scabridens* E. B. Bartram

Pterobryaceae Kindb. Type: *Pterobryon* Hornsch.
Calyptothecium Mitt., *Cryptogonium* (Müll. Hal.) Hampe, *Henicodium* (Müll. Hal.) Kindb., *Hildebrandtiella* Müll. Hal., *Horikawaea* Nog., *Jaegerina* Müll. Hal., *Micralsopsis* W. R. Buck, *Muellerobryum* M. Fleisch., *Neolindbergia* M. Fleisch., *Orthorrhynchidium* Renauld & Cardot, *Orthostichidium* Dusén, *Orthostichopsis* Broth., *Osterwaldiella* Broth., *Penzigiella* M. Fleisch., *Pireella* Cardot, *Pseudopterobryum* Broth., *Pterobryidium* Broth. & Watts, *Pterobryon* Hornsch., *Pterobryopsis* M. Fleisch., *Pulchrinodus* B. H. Allen, *Renauldia* Müll. Hal., *Rhabdodontium* Broth., *Spriridentopsis* Broth., *Symphysodon* Dozy & Molk., *Symphysodontella* M. Fleisch.

Phyllogoniaceae Kindb. Type: *Phyllogonium* Brid.

Orthorrhynchiaceae S. H. Lin Type: *Orthorrhynchium* Reichardt

Lepyrodontaceae Broth. Type: *Lepyrodon* Hampe

Neckeraceae Schimp. Type: *Neckera* Hedw.
Baldwiniella M. Fleisch., *Bissetia* Broth., *Bryolawtonia* D. H. Norris & Enroth, *Caduciella* Enroth, *Crassiphyllum* Ochyra, *Cryptoleptodon* Renauld & Cardot, *Curvicladium* Enroth, *Dixonia* Horik. & Ando, *Dolichomitra* Broth., *Handeliobryum* Broth., *Himantocladium* (Mitt.) M. Fleisch., *Homalia* (Brid.) Bruch & Schimp., *Homaliadelphus* Dixon & P. de la Varde, *Homaliodendron* M. Fleisch., *Hydrocryphaea* Dixon, *Isodrepanium* (Mitt.) E. Britton, *Metaneckera* Steere, *Neckera* Hedw., *Neckeropsis* Reichardt, *Neomacounia* Ireland, *Noguchiodendron* Ninh & Pócs, *Pendulothecium* Enroth & S. He, *Pinnatella* M. Fleisch., *Porotrichodendron* M. Fleisch., *Porotrichopsis* Broth. & Herzog, *Porotrichum* (Brid.) Hampe, *Thamnobryum* Nieuwl., *Touwia* Ochyra

Appendix 1. Continued.

Echinodiaceae Broth. Type: *Echinodium* Jur.
Leptodontaceae Schimp. Type: *Leptodon* D. Mohr
Alsia Sull., *Forsstroemia* Lindb., *Leptodon* D. Mohr, *Taiwanobryum* Nog.
Lembophyllaceae Broth. Type: *Lembophyllum* Lindb.
Acrocladium Mitt., *Bestia* Broth., *Camptochaete* Reichardt, *Dolichomitriopsis* S. Okamura, *Fallaciella* H. A. Crum, *Fifea* H. A. Crum, *Isothecium* Brid., *Lembophyllum* Lindb., *Neobarbella* Nog., *Orthostichella* Müll. Hal., *Pilotrichella* (Müll. Hal.) Besch., *Weymouthia* Broth.
Myuriaceae M. Fleisch. Type: *Myurium* Schimp.
Eumyurium Nog., *Myurium* Schimp., *Oedicladium* Mitt., *Palisadula* Toyama
Anomodontaceae Kindb. Type: *Anomodon* Hook. & Taylor
Anomodon Hook. & Taylor, *Bryonorrisia* L. R. Stark & W. R. Buck, *Chileobryon* Enroth, *Curviramea* H. A. Crum, *Haplohymenium* Dozy & Molk., *Herpetineuron* (Müll. Hal.) Cardot, *Schwetschkeopsis* Broth.
Theliaceae (Broth.) M. Fleisch. Type: *Thelia* Sull.
Microtheciellaceae H. A. Mill. & A. J. Harr. Type: *Microtheciella* Dixon
Sorapillaceae M. Fleisch. Type: *Sorapilla* Spruce & Mitt.

DIVERSIFICATION OF PEATMOSSES: A PHYLOGENETIC APPROACH[1]

A. Jonathan Shaw,[2] *Cymon J. Cox,*[2] *and Iwona Melosik*[3]

Abstract

Some of the most intriguing questions in evolutionary biology relate to variation in diversification rates (and present diversity) among clades, and through history. Phylogenetic tree shape can provide insights into temporal patterns in biodiversification. Lineage-through-time (LTT) plots provide a quantitative description of tree shape allowing for statistical comparisons of alternative diversification models. In this study, we generate LTT plots from phylogenetic trees for *Sphagnum* based on nr ITS and cpDNA *trn*L-*trn*F nucleotide sequences. Analyses of LTT plots indicate that the diversification rate of *Sphagnum* has decreased through time. The effects of phylogenetic uncertainty on LTT plots were investigated by sampling 20 random trees from the 95% Bayesian posterior probability space, and the effects on inferences about diversification were insignificant. Unless *Sphagnum* is twice as diverse at the species level as any recent systematist has suggested, the apparent decreasing diversification rate is unlikely to be an artifact of incomplete sampling.

Key words: density-dependent diversification, diversification, ITS, Lineages-through-time plots, phylogenetics, *Sphagnum*, *trn*L-*trn*F.

Phylogenetic analyses have become central to a broad range of biological research including (but not limited to) systematics, population genetics, molecular evolution, agriculture, epidemiology, and conservation biology. Most commonly, the topologies of phylogenetic trees are used to infer ancestor-descendent relationships among taxonomic units (which might range from gene copies to phyla). A good example is the recent and ongoing major initiative to resolve the Tree-of-Life (Sudgen et al., 2003). There has been stunning progress toward resolution of the moss Tree-of-Life during the last five years, and we are now in a position to begin asking questions about how that tree has grown. We can use phylogenetic trees to clarify the processes underlying moss *diversification*.

Diversification rates depend on the rates of two processes: cladogenesis (speciation) and extinction. If the rate of cladogenesis exceeds the rate of extinction, a positive diversification rate results; if extinction rates exceed cladogenesis, then a negative rate of diversification results; and if this persists, disappearance of the whole clade is inevitable. The expected mean number of species is constant through time if rates of cladogenesis and extinction are equal. Studies of diversification have traditionally fallen under the rubric of paleontology, with its explicit time element and its window into the existence of now extinct lineages (e.g., Stanley, 1979; Raup, 1985; Benton, 1997). With the continued development and refinement of techniques for molecular phylogenetics, however, it is widely acknowledged that inferences about diversification patterns can also be made from phylogenetic analyses of extant taxa (Hey, 1992; Nee et al., 1992, 1994a; Harvey et al., 1994). Nee et al. (1994a) and Harvey et al. (1994) discussed the relationship between actual and reconstructed phylogenies (the former including both extant and extinct lineages while the latter, obviously, includes only those lineages that are extant). Modern phylogeneticists deal with reconstructed phylogenies. At any given time, the actual phylogeny will include as many or more lineages than the reconstructed phylogeny. If there has been no extinction, the two are equivalent.

The two central questions in this still-emerging field pertain to differences in rates of diversification among clades and variation in diversification rates over time within a single clade. Indeed, one of the most intriguing questions about the world's biodiversity is why some lineages are so speciose while others are relatively depauperate. Why are there so many species of *Fissidens* and so few of *Scopelophila* (Crosby et al., 1999)? In addition to differences in rates of diversification among lineages, it is firmly established that individual groups have

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gone through periods of faster and slower diversification during their history. This conclusion is supported by both paleontological data (e.g., Sepkoski, 1978, 1979) and phylogenetic analyses (e.g., Sanderson & Donoghue, 1994; Sanderson, 1997), and raises interesting questions about the factors underlying rates of diversification.

In order to test hypotheses about heterogeneity in diversification rates, either among lineages or through time, appropriate null hypotheses need to be formulated. The appropriate null hypothesis for tests of among-clade heterogeneity in diversification rates is a pattern in which rates are homogeneous. A number of statistical approaches have been formulated to test departures from the null hypothesis (Hey, 1992; Kirkpatrick & Slatkin, 1993; Purvis et al., 1995; Sanderson & Donoghue, 1996).

The present study focuses on the diversification rate characterizing a monophyletic group (*Sphagnum*) through time rather than comparing rates among clades within that group. The basic question is whether diversification rate has remained constant through the phylogenetic history of peatmosses. The two issues are not entirely independent, of course, since a substantial speedup (or slowdown) in diversification within a subclade of peatmosses could cause a detectable change in average rate for the group as a whole. The net result of heterogeneity in rates of diversification within subclades could be complex. Consider, for example, a clade made up of two lineages with high and low rates of speciation, respectively. The average diversification rate will increase through time because the high speciation rate lineage will make up a greater and greater percentage of the total species. The same would be true if the two lineages differ in extinction rates (Paradis, pers. comm.). Diversification rate heterogeneity among clades within *Sphagnum* will be addressed in a subsequent publication. Here we smooth any rate heterogeneity among lineages using Sanderson's (1997) algorithm.

The most commonly used null hypothesis for tests of temporal heterogeneity in diversification rate is rate constancy with stochastic variation (such that predictions are probabilistic rather than deterministic—Raup, 1985; Hey, 1992; Nee et al., 1992; Harvey et al., 1994). Hypothesis testing involves specifying a model of diversification, making predictions about phylogenetic tree shape based on that model, and testing for significant departures from those predictions. The general approach was lucidly outlined by Raup (1985), among others.

A constant-rate model of diversification means that each lineage has some probability of bifurcating (cladogenesis) or going extinct per unit time, and that this probability remains constant through time. Hey (1992) and Wollenberg et al. (1996) utilized models in which the total number of lineages remains constant such that any time a lineage bifurcates, another lineage is assumed to go extinct. In contrast to this equilibrium model of diversity, most authors (e.g., Harvey et al., 1994; Nee et al., 1994a, b; Paradis, 1998a) utilize a more general model that assumes an exponential increase in (actual) lineages. The basic form of this model specifies the expected number of lineages at time t as:

$$m_t = ae^{(\lambda-\mu)t}$$

where m_t is the expected number of lineages at time t, a is the beginning number of lineages, λ is the branching probability, and μ is the probability of extinction. Once this null model is specified, predictions about tree shape are readily derived. A commonly utilized prediction, for example, relates to the distribution of expected branching times (internode lengths in a molecular phylogeny), and tests of constant diversification rates compare observed internode intervals to this null expectation. Other statistics that can be specified under a constant rate, exponential model of diversification include the expected number of lineages at specified times (see equation above), the distribution of clade longevities (expected times to extinction), and expectations about the number of descendents left by clades (Raup, 1985).

One way to represent the shape and branching pattern of a phylogenetic tree is a lineages-through-time (LTT) plot (Nee et al., 1992, 1994a, b; Harvey et al., 1994). An exponential, constant diversification-rate phylogeny yields a straight line when the log of the number of lineages is plotted against time. Harvey et al. (1994) illustrated the expected forms of LTT plots for the actual phylogeny (including all extinct as well as extant lineages) and also for a phylogeny reconstructed from extant taxa only. The number of lineages in the actual and reconstructed plots start out and end at the same points, but in between the number of lineages in the reconstructed LTT plot is less than the actual number because of lineages that have gone extinct. The two lines have the same expected slope for much of their extents, but the reconstructed plot characteristically exhibits an upturn with higher slope near the present time. This upturn, which Nee et al. (1994b) referred to as the pull of the present, results from the fact that when the rate of lineage origination (cladogenesis, λ) is greater than lineage extinction (μ), the most recently formed lineages will not have had time to go extinct. The

greater the difference between λ and μ, the more obvious is the apparent recent increase in diversification rate. Thus, whereas the slope of the main part of the LTT plot reflects the diversification rate (λ − μ), the slope of the line near the present approaches the rate of cladogenesis itself (λ).

LTT plots provide a graphical representation of the null hypothesis that the diversification rate has been constant through the time frame encompassed by the phylogeny. Several statistical tests have been proposed to assess whether the null model of constant diversification should be rejected (Nee et al., 1992; Purvis et al., 1995). Paradis (1997) developed statistical tests based on survival analysis (times to extinction) to compare three models of diversification: constant exponential diversification (his model A), gradually increasing or decreasing rates of diversification (his model B), and a pattern with two discretely different rates at different times during a clade's history (his model C). Assessments of which model best fit the data can be made using likelihood ratio tests (LR) and the Akaike Information Criterion (AIC) (Paradis, 1997).

Shaw et al. (2003a) used the statistical method described by Paradis (1997), implemented in his software DIVERSI (Paradis, 2000), to test which model (A, B, C, as above) best fit the diversification patterns in two orders of pleurocarpous mosses, the Hypnales and Hookeriales. Sampling of taxa from these two orders, while arguably random (Shaw et al., 2003a), was obviously very incomplete at the species level. Indeed, Shaw et al. estimated that their samples constituted about 7% of all extant species of Hookeriales and 4% of extant Hypnales. Nee et al. (1994a, b) had previously shown that incomplete sampling results in LTT plots that appear to illustrate a gradually decreasing rate of diversification over time. That is, incomplete sampling could lead to a false rejection of the null hypothesis that the diversification rate has been constant. Taking this sampling effect into consideration by simulating phylogenies under constant diversification, but with comparably incomplete sampling (4% & 7%) of taxa from the reconstructed phylogenies, Shaw et al. rejected model A (constant diversification) in favor of model C (two different diversification rates) for the Hypnales. Although the LTT plot characterizing Hookeriales *appeared* to best illustrate a decreasing rate of diversification (i.e., a best fit for model B), simulations studies showed that such LTT plots could be produced by incomplete sampling from a large clade having undergone constant diversification. Thus, model A could not be rejected.

Although LTT plots can provide exciting insights into the diversification process, a number of factors can lead to ambiguity and possibly erroneous evolutionary interpretations. For the Hookeriales, incomplete sampling could cause the erroneous rejection of model A, as described above. Other potential pitfalls in the use of LTT plots to assess diversification rates include (1) lack of a molecular clock such that branch lengths do not provide a good proxy for time; (2) non-random sampling of taxa to generate the phylogeny; (3) uncertainty in the phylogenetic reconstruction; and (4) locus-specific effects. This paper describes analyses of diversification rates in the peatmosses (*Sphagnum*). The peatmosses are also used as a case study to further explore the effects of incomplete sampling on inferences about diversification, and also the impact of phylogenetic uncertainty.

Materials and Methods

the data set

The phylogenetic and diversification analyses were based on a data set consisting of *trn*L-*trn*F (cpDNA) and ITS (nrDNA) sequences from 240 samples of *Sphagnum*. The data set utilized here is a subset of that used in the analyses of present, worldwide diversity patterns in peatmosses described by Shaw et al. (2003b). For the present analyses, samples that were identical with others for both ITS and *trn*L-*trn*F were deleted so that all samples in the current data set are unique in terms of nucleotide sequences. Collection localities, voucher information, and GenBank numbers for all the sequences used for analyses in this paper were provided in Shaw et al. (2003b). Species identifications and country of origin are summarized for the 240 samples in Table 1. Extraction of DNA and amplification of the genomic regions was accomplished according to the protocols described in Shaw (2000).

The effect of incomplete but random sampling of extant taxa from a clade on the shape of the derived LTT plot for that clade has been amply documented, and it is important that the relative exhaustiveness of sampling be taken into consideration lest spurious inferences be made about changing rates of diversification (Nee et al., 1994b; Pybus & Harvey, 2000; Shaw et al., 2003a). Nee et al. (1994b) discussed the issue as one of sampling *lineages* for phylogenetic analyses and the construction of LTT plots. Subsequent authors, however, have stated the goal of these analyses as estimating species diversification rates (e.g., Sanderson & Donoghue, 1994; Pybus & Harvey, 2000), and (implicitly or explic-

itly) couched the issue of complete sampling in terms of including all known *species* in a clade.

There are two problems surrounding the assessment of sampling completeness. One is that the definition of species is hardly an objective one; what one taxonomist might interpret as a single species, another might interpret as two or five or ten. *Sphagnum* is arguably one of the most difficult genera of mosses in which to define species, so this problem is especially acute. Another is that many or most species of *Sphagnum*, like almost all other species of plants, animals, and fungi (Avise, 2000), contain phylogenetically structured variation within them. So what constitutes "complete" sampling? There is clearly no objectively correct answer.

The sample of *Sphagnum* collections included in the present analyses are reasonably close to complete at the species level. Samples of nearly all generally recognized Northern Hemisphere species are included. Samples from tropical latitudes and Southern Hemisphere regions were included on an opportunistic basis; almost every available sample that was collected recently enough for successful amplification of the genomic regions on which the phylogeny was based was included. There are some unrepresented species, especially from Australasia and Africa, that were recognized by Fife (1996), Seppelt (2000), and/or Eddy (1977, 1985), but these are relatively few. The data set considered herein does include samples from these regions. A large percentage of South American species described by Crum (e.g., 1987a, b, 1989, 1990a, b, 1992a, b, 1993a, b, 1994, 1995a, b, 2001b) are included in the current analyses (see Table 1), many on the basis of molecular data derived from type material (with permission).

We considered our sample of *Sphagnum* for these analyses to be nearly complete at the species level, even if not absolutely exhaustive. Moreover, our sampling included numerous populations that according to current taxonomic concepts would be considered conspecific. This is especially true of Northern Hemisphere taxa for which ample material was available, but was also true of some tropical and Southern Hemisphere regions. Sampling included populations from all continents, with broad geographic representation within most continents. Thus, our a priori interpretation was that the 240 samples included in analyses represent, for all practical purposes, a complete sampling for the genus.

PHYLOGENETIC ANALYSES

Phylogenetic relationships were reconstructed using maximum parsimony and Bayesian inference. Parsimony analyses were constrained to the topology resolved by a 16-gene backbone analysis in which relationships among the infrageneric sections were resolved with strong support (Shaw et al., 2003c). Genomic sampling included 7 nuclear (ITS, *LEAFY1*, *LEAFY2*, 26S, 3 anonymous regions), 6 chloroplast (*psb*A, *psb*T, *rbc*L, *rps*4, *trn*G, *trn*L), and 3 mitochondrial (*nad1*, *nad5*, and *nad7*) loci. There were 20,000 trees retained from a heuristic search with 300 random-addition replicates.

Bayesian analyses were performed using MrBayes 3.04 beta (Huelsenbeck & Ronquist, 2002). Analyses were conducted with six runs, each with 2,000,000 generations using uniform priors. The optimal substitution model was determined by hierarchical likelihood ratio tests with the aid of MrModeltest 1.1b (Nylander, 2002). Model parameters including trees were sampled every 100th generation. Trees of the burnin (i.e., those before the chain reached stationarity) for each run were excluded from the tree set, and the remaining trees from each run were combined to form the full sample assumed to be representative of the posterior probability (p.p.) distribution.

CONSTRUCTION OF LTT PLOTS FROM RECONSTRUCTED PHYLOGENIES

The reconstruction of *Sphagnum* phylogeny departed significantly from a global clocklike pattern (LTT = 855.398; 241 d.f.; $P \ll 0.001$); hence substitution rates and time are confounded as causal mechanisms underlying branch lengths. We utilized Sanderson's (1997) nonparametric rate smoothing (NPRS) procedure, implemented in his program r8s (version 1.05 beta), to tease these two factors apart. The method minimizes changes in local substitution rate on the phylogenetic tree such that rates are smoothed across the whole tree. Rate smoothing allows branch lengths to be used as proxies for time in the absence of a molecular clock.

The root node, or most recent common ancestor (MRCA), of the NPRS tree was set to 100 time units. The Powell algorithm under the NPRS model was used to optimize branch lengths with ten random initializations each with ten repeated perturbations. The number of lineages through time in the NPRS tree was calculated in GENIE (Pybus & Rambaut, 2002). This procedure was repeated for a random sample of 10 most parsimonious (MP) trees and for 20 trees randomly sampled from the 95% posterior probability space recovered from the Bayesian analyses. From these replicates, mean LTT plots were obtained, along with standard de-

Table 1. Samples included in the phylogenetic reconstruction on which the present diversification analyses are based. Taxa in this study are a subset of the samples included in Shaw et al. (2003b). GenBank numbers and more complete accession information for the samples are provided in that publication.

Taxon	Location
S. aciphyllum Müll. Hat.	Brazil
S. acutirameum H. A. Crum	Brazil
S. affine Renauld & Cardot	India
S. affine	U.S.A.
S. africanum Welw. & Duby	South Africa
S. africanum	South Africa
S. alegrense Warnst.	Australia
S. amoenoides H. A. Crum	Brazil
S. andersonianum R. E. Andrus	England
S. andersonianum	Ireland
S. andersonianum	U.S.A.
S. andersonianum	U.S.A.
S. andersonianum	U.S.A.
S. angermanicum Melin	U.S.A.
S. angermanicum	U.S.A.
S. angustifolium (Warnst.) C. E. O. Jensen	Norway
S. annulatum Warnst.	Japan
S. annulatum	Norway
S. aongstroemii C. Hartum	Norway
S. aongstroemii	Russia
S. aongstroemii	Russia
S. arcticum Flatberg & Frisvoll	Norway
S. aureum C. B. McQueen	Panama
S. auriculatum Schimp.	Norway
S. auriculatum	Spain
S. austinii Sull.	Canada
S. austinii	Norway
S. austinii	U.S.A.
S. austro-americanum H. A. Crum	Colombia
S. azuayense H. A. Crum	Ecuador
S. bartlettianum Warnst.	U.S.A.
S. bartlettianum	U.S.A.
S. bartlettianum	U.S.A.
S. bartlettianum	U.S.A.
S. billbuckii H. A. Crum	Brazil
S. boliviae Warnst.	Bolivia
S. bordasii Besch.	South Africa
S. boyacanum H. A. Crum	Colombia
S. brevirameum Warnst.	Brazil
S. buckianum H. A. Crum	Brazil
S. calymmatophyllum Warnst. & Cadot	Japan
S. capense Hornsch.	Malawi
S. capense	South Africa
S. capillifolium (F. W. Weiss) Schrank	Finland
S. capillifolium	Finland
S. carolinianum R. E. Andrews	U.S.A.

Table 1. Continued.

Taxon	Location
S. centrale C. E. O. Jensen	Romania
S. centrale	U.S.A.
S. compactum Lam. & DC.	Norway
S. compactum	Russia
S. compactum	U.S.A.
S. compactum	U.S.A.
S. contortum H. A. Crum	U.S.A.
S. cristatum Hampe	Australia
S. crumii Schaf.-Verw.	Brazil
S. cuspidatulum Müll. Hal.	India
S. cuspidatulum	Malaysia
S. cuspidatum Müll. Hal.	Phillipines
S. cuspidatum	U.S.A.
S. cymbifolioides Breutel	Australia
S. davidii Warnst.	South Africa
S. denticulatum Brid.	Poland
S. ecuadorense Warnst.	Ecuador
S. exquisitum H. A. Crum	Brazil
S. falcatulum Besch.	Tasmania
S. fimbriatum Wilson	Canada
S. fimbriatum	Hungary
S. fimbriatum	Russia
S. fimbriatum	Russia
S. fimbriatum	U.S.A.
S. fitzgeraldii Renauld & Cardot	U.S.A.
S. flaccidum Dixon ex A. Johnson	Colombia
S. flavicomans (Cardot) Warnst.	Canada
S. flavicomans	Canada
S. flavicomans	Canada
S. flavicomans	U.S.A.
S. flavicomans	U.S.A.
S. flexuosum Dozy & Molk.	U.S.A.
S. fuscum Seppelt & H. A. Crum	Canada
S. fuscum	Japan
S. fuscum	Japan
S. fuscum	U.S.A.
S. fuscum	U.S.A.
S. geraisense H. A. Crum	Brazil
S. girgensohnii Russow	Japan
S. girgensohnii	U.S.A. (NY)
S. girgensohnii	U.S.A.
S. girgensohnii	U.S.A.
S. girgensohnii	U.S.A.
S. gracilescens Michx.	Brazil
S. guwassanense Warnst.	Japan
S. henryense Warnst.	U.S.A.
S. imbricatum Hornsch. ex Russow	Japan
S. inundatum Russow	India
S. inundatum	Norway
S. inundatum	Poland
S. inundatum	Poland
S. inundatum	Poland
S. inundatum	U.S.A.
S. inundatum	U.S.A.
S. itatiaiae Müll. Hal. & Warnst.	Brazil
S. jensenii H. Lindb.	Norway

Table 1. Continued.

Taxon	Location
S. junghuhnianum Dozy & Molk.	Taiwan
S. junghuhnianum	Phillipines
S. keniae Dixon	U.S.A.
S. khasianum Mitt.	China
S. lapazense H. A. Crum	Bolivia
S. laxirameum H. A. Crum	Colombia
S. lenense H. Lindb. ex L. L. Savicz	Russia
S. leonii H. A. Crum	Brazil
S. lescurii Sull.	Belize
S. lescurii	Colombia
S. lescurii	U.S.A.
S. lescurii	U.S.A.
S. lescurii	U.S.A.
S. lescurii	U.S.A.
S. lescurii	U.S.A.
S. lescurii	U.S.A.
S. limbatum Mitt.	Costa Rica
S. lindbergii Schimp.	Canada
S. lindbergii	Norway
S. macrophyllum Berhn. ex Brid.	U.S.A.
S. macrophyllum	U.S.A.
S. magellanicum Brid.	China
S. magellanicum	Japan
S. magellanicum	U.S.A.
S. majus (Russow) C. E. O. Jensen	Norway
S. majus	Norway
S. majus	Norway
S. majus	Poland
S. meridense (Hampe) Müll. Hal.	Bolivia
S. meridense	Bolivia
S. meridense	Honduras
S. meridense	Mexico
S. microcarpum Warst.	U.S.A.
S. microporum Warst. ex Cardot	Japan
S. microporum	Japan
S. molle Sull.	Ireland
S. molle	U.S.A.
S. molle	U.S.A.
S. monzonense Warnst.	Bolivia
S. nemoreum Scop.	Japan
S. novo-zealandicum Mitt.	Australia
S. novo-zealandicum	Australia
S. obtusum Warnst.	U.S.A.
S. ovatum Hampe	Bhutan
S. oxyphyllum Warnst.	Bolivia
S. palustre L.	Bhutan
S. palustre	U.S.A.
S. papillosum Lindb.	Japan
S. papillosum	U.S.A.
S. patens Brid.	Mauritius
S. perfoliatum L. I. Savicz	Canada
S. perichaetiale Hampe	Paraguay
S. perichaetiale	Trinidad

Table 1. Continued.

Taxon	Location
S. perichaetiale	U.S.A.
S. planifolium Müll. Hal.	Equatorial Guinea
S. planifolium	Kenya
S. platyphylloides Warnst.	Brazil
S. platyphyllum (Lindb.) Warnst.	U.S.A.
S. platyphyllum	U.S.A.
S. portoricense Hampe	U.S.A.
S. pulchricoma Müll. Hal.	Bolivia
S. pulchrum (Lindb.) Warnst.	Finland
S. pulchrum	Norway
S. pycnocladulum Müll. Hal.	Malawi
S. pylaesii Brid.	Canada
S. pylaesii	Canada
S. pylaesii	Colombia
S. quinquefarium (Lindb.) Warnst.	U.S.A.
S. quinquefarium	U.S.A.
S. quinquefarium	U.S.A.
S. recurvum P. Beauv.	Japan
S. recurvum	U.S.A.
S. riparium Ångström	Belgium
S. ripense H. A. Crum & W. R. Buck	Brazil
S. rotundatum Müll. Hal. & Warnst.	Brazil
S. rotundatum	Brazil
S. rubellum Wilson	Norway
S. rubellum	Russia
S. rubroflexuosum R. W. Andrus	U.S.A.
S. russowii Warnst.	Russia
S. russowii	U.S.A.
S. sancto-josephense H. A. Crum & Crosby	Ecuador
S. schofieldii H. A. Crum	Canada
S. sericeum Müll. Hal.	Malaysia
S. skyense Flatberg	Scotland
S. sonsonense H. A. Crum	Colombia
S. sparsum Warnst.	Bolivia
S. squarrosum Dixon & Sherrin	Canada
S. squarrosum	India
S. squarrosum	Japan
S. squarrosum	Japan
S. strictum Sull.	Colombia
S. strictum	Cuba
S. strictum	U.S.A.
S. strictum	Uganda
S. strictum	Uganda
S. subditivum H. A. Crum	New Zealand
S. subfulvum Sjors	Japan
S. subfulvum	Norway
S. subfulvum	U.S.A.
S. subfulvum	U.S.A.
S. subhomophyllum H. A. Crum	Brazil
S. subnitens Russow & Warnst.	Luxembourg
S. subsecundum H. A. Crum & W. R. Buck	Belgium
S. subsecundum	Canada

Table 1. Continued.

Taxon	Location
S. subsecundum	Canada
S. subsecundum	Finland
S. subsecundum	Japan
S. subsecundum	New Zealand
S. subsecundum	Poland
S. subsecundum	Poland
S. subsecundum	Poland
S. "subsec"	Uganda
S. sucrei H. A. Crum	Brazil
S. tenellum (Brid.) Brid.	Japan
S. tenellum	Japan
S. tenerum Sull. & Lesq. ex Sull.	U.S.A.
S. tenerum	U.S.A.
S. teres (Schimp.) Ångström	Canada
S. torreyanum Sull.	Canada
S. torreyanum	Canada
S. triparum H. A. Crum	Brazil
S. troendelagicum Flatberg	Norway
S. truncatum Hornsch.	South Africa
S. truncatum	South Africa
S. tundrae Flatberg	Norway
S. viridum Flatberg	Ireland
S. warnstorfii Russow	U.S.A.
S. warnstorfii	U.S.A.
S. warnstorfii	U.S.A.
S. wilfii H. A. Crum	Canada
S. wulfianum Girg.	Canada
S. wulfianum	U.S.A.
S. wulfianum	U.S.A.
S. sp.	Ecuador
S. sp.	Papua New Guinea

viations that reflect phylogenetic uncertainty as represented by the 10 or 20 sampled trees.

The program DIVERSI version 0.20.0 (Paradis, 2000) was used to test alternative models of diversification. Three models were tested: model A specifies a constant rate of diversification (i.e., the expected number of lineages increases at a constant exponential rate); model B specifies that diversification rate has increased or decreased gradually over time; and model C specifies that a change in diversification rate has occurred such that two different rates, distinguished by an abrupt shift at some point in the past, best fit the data. For model B, the parameter β is < 1 when diversification rate increases gradually through time and is > 1 when the rate decreases through time (Paradis, 1997, 2000). Alternative models are evaluated using likelihood ratio (LR) tests and/or the Akaike Information Criterion (AIC; Paradis, 1997). The LR test is appropriate for evaluating models A versus B and A versus C because model A is nested within both models B and C. No such nested relationship exists between models B and C, so using a chi-square distribution to test significance of the LR is inappropriate. Another approach for testing alternative models is the AIC, a likelihood-based metric with explicit penalties for the number of model parameters. The AIC must be used to evaluate non-nested models (Paradis, 1997, 2000). The number of lineages through time data calculated in GENIE (see above) were converted to the DIVERSI format with appropriate corrections for polytomies (see Paradis, 2000). The alternative models of diversification were evaluated for each of the 10 MP trees and 20 Bayesian trees so that the effects of phylogenetic ambiguity on statistical evaluations of diversification rate could be assessed.

SIMULATION OF CONSTANT DIVERSIFICATION-RATE PHYLOGENIES

PHYLOGEN (Rambaut, 2002) was used to generate 100 replicate phylogenies under a constant diversification rate (birth : death = 2 : 1). Simulations were used to compare the empirical LTT plot derived from the 240-sample phylogeny to plots derived from sampling 240 terminals from larger clades. This was done to assess whether the diversification pattern inferred from the real data could reflect incomplete sampling from a larger clade, rather than the actual underlying diversification rate. Simulated phylogenies were generated for clades consisting of 250 to 1000 terminals at intervals of 50. From each of these phylogenies, 240 taxa were sampled at random and LTT plots were generated from these subsamples. In other words, we evaluated the hypotheses that our 240-sample (real) data set represents a random sample from 250, 300, 350, . . . , 1000 actual extant *Sphagnum* lineages.

RESULTS

SPHAGNUM LINEAGES-THROUGH-TIME (LTT) PLOTS

LTT plots for a sample of 20 trees from the Bayesian posterior probability distribution and 10 maximum parsimony trees are shown in Figures 1A and 1B, respectively. Error bars represent standard deviations of estimates for the time (since the root) when the phylogenies had a given number of lineages. Clearly, phylogenetic uncertainty, at least as reflected by this approach to sampling possible trees, has a minimal effect on the shape of LTT plots.

LTT plots from both the Bayesian and parsimony sets of trees exhibit a decreasing slope; i.e., the diversification rate appears to have decreased

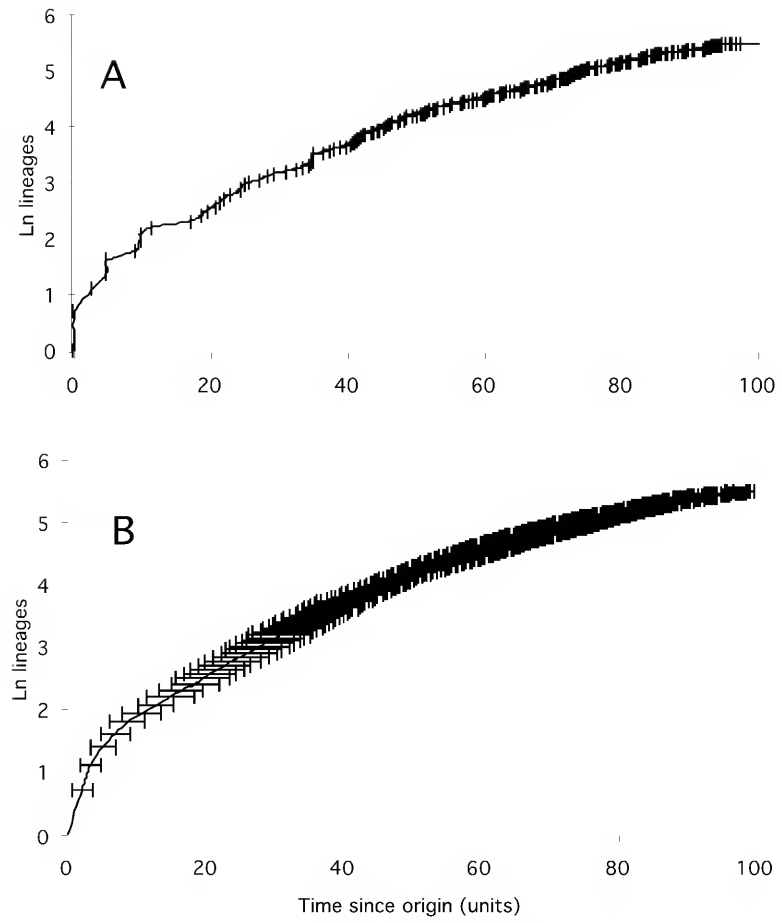

Figure 1. Lineage-through-time (LTT) plots for *Sphagnum* from phylogenetic trees resolved using maximum parsimony (A) and Bayesian inference (B). Standard deviations are based on 10 MP trees in A and 20 Bayesian trees in B.

through time, and phylogenetic ambiguity does not cloud that interpretation. Each of the LTT plots derived from Bayesian trees was subjected to statistical analyses using the method of Paradis (1997, 2000). Nineteen of the 20 trees best fit model B, while one tree best fit model A. Thus, we can reject model A at the 5% level in favor of model B, which specifies a gradually changing rate of diversification. Beta (β) was greater than unity, indicating that the diversification rate has decreased over time.

SIMULATED PHYLOGENIES AND THEIR LTT PLOTS

Since model B could best fit the phylogenetic pattern in *Sphagnum* simply because the data might represent an incomplete sample from a large clade, we simulated phylogenies in which our 240-taxon phylogeny represented a random sample from a larger clade. When LTT plots were constructed for these simulated phylogenies, the effects of sampling are clearly evident (Fig. 2). If our 240 accessions were sampled from a clade that, in fact, had 1000 lineages, the LTT plot would exhibit a decreasing slope similar to that produced by a changing diversification rate even though the underlying phylogeny was characterized by a constant rate of diversification (Fig. 2). As the size of the clade was decreased in the simulations (always generating LTT plots from samples of 240 randomly selected terminals), the plots approached the expected shape characteristic of constant diversification phylogenies. If the 240-taxon sample came from a clade with 300 taxa (Fig. 2, lower right), the LTT plot has the expected shape for a phylogeny with constant diversification, including the characteristic increase in slope near the present (the so-called pull of the present). For total clade sizes above approximately 600 taxa, the LTT plot for a random sample of 240 terminals exhibits a visible decrease in slope near the present (Fig. 2).

Statistical tests of alternative diversification models were conducted on the simulated LTT plots for all total clade sizes between 250 and 1000 terminals, at intervals of 50 (DIVERSI results summarized in Table 2). The results indicate that the statistical tests of Paradis (1997, 2000) are not without ambiguity. The results in Table 2 are from 100 tests executed for 100 randomly selected (simulated) trees. For example, when LTT plots were constructed for 240 terminals sampled from a total clade of 1000, 95% of the plots best fit model B when models A and B were compared using likelihood ratio (LR) tests. Not surprisingly, when only models A and C were compared, neither provided a clear best fit to the data. The Aikake Information Criterion was also used to compare the models and model B again provided a strong fit when A and B were compared. When models B and C were compared, model B provided a best fit for 93% of the trees, just below the 5% significance level. When all three models were compared using the AIC, model B provided a best fit for 93% of the 100 trees. In this case, the LR and AIC provided congruent inferences; constant rate, simulated phylogenies, with only 240 terminals sampled from 1000 total, yield LTT plots that appear to reflect decreasing diversification rates, but this effect is simply due to incomplete sampling of extant taxa.

Sampling 240 terminals from phylogenies simulated with constant rates of diversification can yield LTT plots from which a decreasing rate of diversification (model B) provide a best fit (Table 2). Focusing on the LR test results, it is clear that the percentage of trees that best fit model B, rather than the (correct) model A, decreases as the total clade size decreases (i.e., as the sample of 240 taxa represents a larger and larger percentage of the total extant lineages). The trend is approximate because of the stochastic nature of the simulations, but the pattern is clear. A statistical threshold is reached when the 240 samples came from a phylogeny with 500 total (simulated) taxa. At this point, less than 5% of the LTT plots best fit model B, while 97%

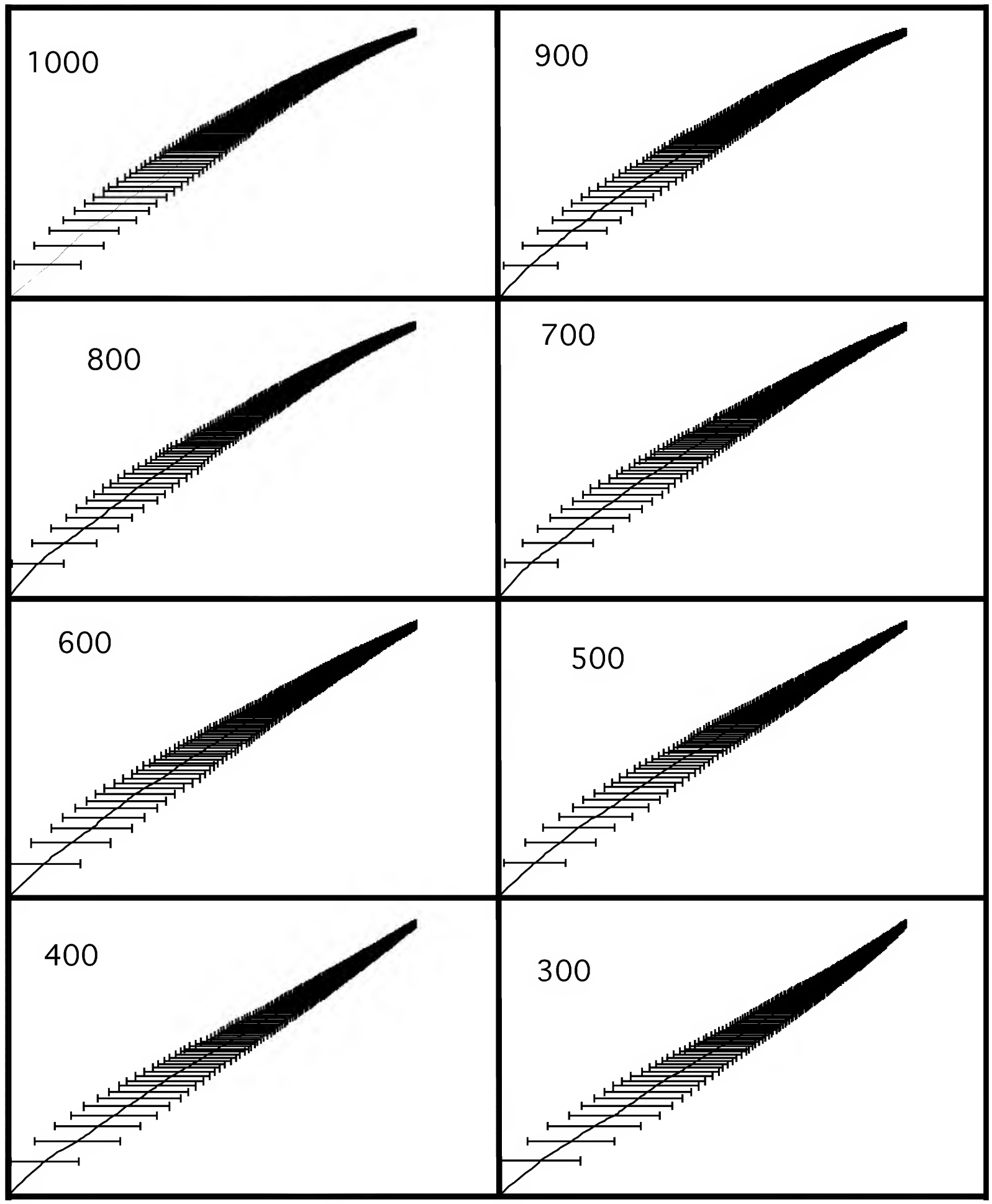

Figure 2. Lineage-through-time (LTT) plots from simulated phylogenetic trees generated under a model of constant exponential diversification. Each LTT plot was produced from a phylogenetic tree with 240 terminal taxa, sampled randomly from a phylogeny with 1000, 900, 800, . . . , 300 taxa (numbers in upper left of each frame).

best fit the model A, under which the phylogenies were actually simulated. Extrapolating to *Sphagnum*, if our 240 taxon data set represents a sample from more than 500 extant Sphagna, we can expect to obtain a best fit for model B, even if the diversification rate has been constant, simply because of the incomplete sampling. If, on the other hand, there are fewer than 500 extant Sphagna, then our empirical LTT plot (Fig. 1) is unlikely to be an artifact of sampling incompleteness, and may truly reflect a decreasing rate of diversification.

For phylogenies simulated with fewer than 500

Table 2. Results of diversification model tests using DIVERSI (Paradis, 2000). The top row (bold) gives the results of tests on 20 trees from the 95% Bayesian posterior probability space from phylogenetic analyses of *Sphagnum*. Other entries represent the results of 100 simulations. 240 terminals were randomly pruned from phylogenies simulated with a constant rate of diversification (birth minus death = 2 − 1) and with total clade size (N). LR = likelihood ratio test; AIC = Akaike Information Criterion. Integers are percentages of the 100 simulated trees of each size in which one model of diversification was favored. Model A = constant diversification, model B = gradually changing rate of diversification, model C = two different diversification rates (i.e., an abrupt change in rate). For likelihood ratio tests, model A (the null hypothesis) was favored unless an alternative model made the data significantly more likely.

Taxa (N)	LR A * B	LR A * C	AIC A * B	AIC B * C	AIC A * B * C	Beta (β)
240 samples (*Sphagnum*)	**A: 95 B: 5**	**A: 0 C: 100**	**A: 5 B: 95**	**B: 90 C: 10**	**A: 0 B: 90 C: 10**	
1000 taxa	A: 5 B: 95	A: 43 C: 57	A: 1 B: 99	B: 93 C: 7	A: 0 B: 93 C: 7	1.204
950 taxa	A: 12 B: 88	A: 43 C: 57	A: 4 B: 96	B: 80 C: 20	A: 2 B: 80 C: 18	1.188
900 taxa	A: 18 B: 82	A: 54 C: 46	A: 3 B: 97	B: 84 C: 16	A: 2 B: 82 C: 16	1.167
850 taxa	A: 23 B: 77	A: 64 C: 36	A: 9 B: 91	B: 83 C: 17	A: 3 B: 81 C: 16	1.158
800 taxa	A: 25 B: 75	A: 65 C: 35	A: 12 B: 88	B: 82 C: 18	A: 11 B: 76 C: 13	1.145
750 taxa	A: 43 B: 57	A: 66 C: 34	A: 28 B: 72	B: 72 C: 28	A: 17 B: 59 C: 24	1.119
700 taxa	A: 51 B: 49	A: 79 C: 21	A: 27 B: 73	B: 73 C: 27	A: 20 B: 61 C: 19	1.106
650 taxa	A: 67 B: 33	A: 76 C: 24	A: 37 B: 63	B: 63 C: 37	A: 26 B: 50 C: 24	1.090
600 taxa	A: 75 B: 25	A: 85 C: 15	A: 55 B: 45	B: 58 C: 42	A: 47 B: 32 C: 21	1.070
550 taxa	A: 85 B: 15	A: 88 C: 12	A: 72 B: 28	B: 46 C: 54	A: 52 B: 19 C: 29	1.045
500 taxa	A: 97 B: 3	A: 90 C: 10	A: 85 B: 15	B: 42 C: 58	A: 65 B: 10 C: 25	1.025
450 taxa	A: 95 B: 5	A: 93 C: 7	A: 86 B: 14	B: 51 C: 49	A: 66 B: 14 C: 20	1.004
400 taxa	A: 96 B: 4	A: 98 C: 2	A: 78 B: 22	B: 54 C: 46	A: 71 B: 20 C: 9	0.982
350 taxa	A: 85 B: 15	A: 98 C: 2	A: 70 B: 30	B: 72 C: 28	A: 63 B: 27 C: 10	0.951
300 taxa	A: 66 B: 34	A: 97 C: 3	A: 53 B: 47	B: 77 C: 23	A: 45 B: 46 C: 9	0.993
250 taxa	A: 32 B: 68	A: 93 C: 7	A: 16 B: 84	B: 94 C: 6	A: 14 B: 84 C: 2	0.887

taxa, the percentages of simulated trees that appear to best-fit model B begin to increase again (Table 2). Thus, for simulated phylogenies with 400 taxa (from which 240 were sampled to produce the LTT plots), only 4% of the plots best fit model B. Model A, the correct pattern, is still favored by LR tests 96% of the time. But for 360 simulated taxa, LH tests indicate that 10% of the trees best fit model B, and that percentage increases as the number of simulated taxa decrease (and sampling [of 240 terminals] becomes more complete). This seems to be paradoxical, until attention is turned to values of β (Table 2). Large trees (i.e., less than 500 simulated taxa) yield values for β that are > 1, indicating a decreasing rate of diversification. At about that 500-taxon threshold (see above), the LTT plot is nearly straight and model A provides an unambiguous best fit to the data. But sampling 240 terminals from smaller clades, such as one that was simulated with a total of 300 taxa (Fig. 2), the pull of the future results in an increase in slope near the present and DIVERSI estimates values for β that are < 1, implying an increasing rate of diversification, at least for some of the simulated trees (34% in the case of 300 taxa; Table 2). DIVERSI is seemingly fooled by the upturn in *apparent* diversification rate in exhaustively or nearly exhaustively sampled clades that have, in fact, diversified by a constant rate.

Analyses presented in Table 2 also indicate that the LR and AIC criteria differ in their efficacy for distinguishing the three models of diversification. There is no sampling threshold based on the AIC, as we observed using LR tests, where we can infer that sampling is complete enough to make unambiguous inferences about diversification. When 240 terminals were sampled from 500 simulated taxa, 15% of the LTT plots still best fit model B using the AIC (vs. 3% based on LR tests). When the simulated phylogeny had 450 taxa, only 5% of the LTT plots still (incorrectly) fit model B using LR tests, but the AIC favored model B 14% of the time (Table 2). When all three models were compared using the AIC, DIVERSI results were ambiguous at every sampling level.

Discussion

LTT plots for *Sphagnum* imply that the genus has undergone a decreasing rate of diversification during its relatively recent history. As we and others (Nee et al., 1994b; Shaw et al., 2003a) have shown, LTT plots of this shape (sigmoidal) can reflect incomplete sampling from a clade that has, in fact, experienced a constant exponential rate of diversification. The question is does the apparent decrease in diversification rate in *Sphagnum* result from an artifact of sampling, or has diversification rate really decreased over time?

Most discussions of sampling completeness in the context of estimating diversification rates have focused on sampling at the level of species. In order to assess how complete our sampling was, we need an estimate of worldwide species richness in *Sphagnum*. There is little consensus on what constitutes a species in *Sphagnum* (compare, for example, Lindberg, 1882; Warnstorf, 1911; Andrews, 1913; Crum, 1984; Flatberg, 2002). In their worldwide checklist of mosses, Crosby et al. (1999) listed 287 species of *Sphagnum*, but many of these are poorly known and some are represented by the type specimen only. Crum (2001a) estimated the number of Sphagna as approximately 250 species, while Andrus (2003, pers. comm.) estimated 200 species and Flatberg (2003, pers. comm.) estimated 200–210. There seems to be a consensus that the number is between 200 and 300. If that is accurate, our sampling of 240 accessions represents a large percentage of the total species in this clade. Our sample includes multiple populations of some species (see Table 1), but the sampling is worldwide and appears to include most of the diversity at the species level.

Simulations presented in this paper indicate that if there are no more than 500 species of *Sphagnum* total, our sample should be sufficient to reject the null hypothesis of a constant diversification rate for the genus. Judging from all estimates of species richness that have been published (or communicated to us personally by experts working on peatmoss systematics), we conclude that sampling incompleteness is not the basis for the apparent decrease in diversification rate evidenced by the *Sphagnum* LTT plot.

It can be argued that there is nothing inherently more real about species than about clades within those groups we define as species. Pybus et al. (2003) noted the same problem in their analysis of diversification rate for "species" in the viral genus *Flavivirus* (Flaviviridae), which includes such human pathogens as the yellow fever virus, the West Nile virus, and the dengue virus. They estimated the total size of the *Flavivirus* clade based on the reconstructed phylogeny for a sample of viral accessions (specifically, the distribution of internode lengths in that phylogeny). They acknowledged that, because reproductively isolated units are difficult to define in viruses, their estimate "represents the number of 'species-level' viral lineages in the clade, however such lineages are defined" (Pybus

et al., 2002: 885). Even in groups for which defining species is less problematic, the well-established existence of infraspecific clades raises questions about what constitutes complete sampling in studies of diversification.

If we define the units for which we wish to estimate diversification rates as clades rather than species, there is no realistic hope of gathering complete data. The number of lineages within a group has as an upper bound the number of individual organisms in that clade. However, there is theoretical justification for considering biological species (i.e., reproductively isolated units) as the units for diversification studies. Indeed, evolutionary inferences from tree shape are grounded in fundamentally different models for infra- versus interspecific samples. Reconstructed phylogenies for samples from a single (interbreeding) population are interpreted in terms of coalescence models (e.g., Holmes et al., 1995) and tree shape reflects demographic processes such as changes in population size (or selection). Null expectations are derived from the coalescent models, and incomplete sampling is not an issue since coalescence models assume a small sample from a large population. The null expectations for diversification studies, on the other hand, are derived from birth-death models and do not include any expectation of interbreeding (hybridization, in this context). Reproductive isolation is the (admittedly fuzzy) boundary across which coalescent versus diversification models apply.

Returning to the question regarding *Sphagnum*, what constitutes complete sampling? Unfortunately, we have very little information about mating patterns in natural populations of *Sphagnum*, nor about reproductive compatibility among different morphospecies. Evidence is mounting that reticulate evolution, both among closely related species, and occasionally between more distant congeners, may be an important evolutionary process in peatmosses (Cronberg, 1996; Såstad et al., 1999, 2000, 2001; Shaw & Goffinet, 2000; Cronberg & Natcheva, 2002). While we acknowledge that our sampling is probably not exhaustive at the species level, the simulations presented here suggest that the true number of *Sphagnum* species would need to be at least twice the number included in our sample for our results to reflect sampling incompleteness rather than a slowdown in diversification rate. Our strategy was to sample populations as extensively as possible and not try to rigorously demarcate the limits of species. Our sampling did include populations that according to many current concepts of taxonomic species constitute infraspecific sampling. It is noteworthy that the inclusion of many such terminals in the analysis would have the effect of increasing the number of recent lineages, yet the diversification results suggest just the opposite: a lowered rate of recent cladogenesis. Thus, if anything, our sampling was biased in favor of a conclusion contrary to the pattern we infer from the *Sphagnum* phylogeny. Furthermore, our "infraspecific" sampling included both boreal and Neotropical populations. The latter are significant because Crum (e.g., 1987a, b, 1989, 1990a, b, 1992a, b, 1993a, b, 1994, 1995a, b, 2001b) has described many species from South America, suggesting that the Neotropics may be a hotspot of *Sphagnum* evolution, yet analyses of molecular variation among samples from South America indicate no great abundance of (nucleotide) diversity there (Shaw et al., 2003b). One possible explanation for that seeming contradiction is that *Sphagnum* has recently radiated in South America, producing many "species" that are very closely related on a molecular level. The extent to which DNA-based phylogenetic structure corresponds to genetically-based morphological lineages is of course still unknown.

Other potential sources of error in diversification studies include (but are not limited to) phylogenetic ambiguity, non-clocklike evolution, non-random taxon sampling (discussed above), and locus-specific effects. Our sampling of equally most parsimonious trees, and most likely trees from the Bayesian posterior probability space, suggest that phylogenetic ambiguity may not be a serious problem. The most optimal trees gathered from both methods had relatively similar distributions of internode lengths. Standard deviations on the LTT plots (Fig. 1) are surprisingly small although samples sizes were not large.

Sanderson's non-parametric rate smoothing (NPRS) was used here to correct for non-clocklike evolution in the original data. The conceptual basis for rate smoothing is the idea that nucleotide substitution rates tend to be inherited, and NPRS minimizes abrupt changes in branch length at nodes. Recent work suggests that heritability of cladogenesis rates may well be a general pattern (Savolainen et al., 2002). Sanderson's NPRS method has been used in other studies of diversification (Pybus et al., 2003) so that relative time could be inferred from branch lengths. Pybus and Harvey (2000) introduced other statistics to test for diversification rate heterogeneity among lineages within a larger clade, and through time for the clade as a whole.

Possible locus-specific effects were not pursued in this study, but warrant future investigation. There must be sufficient phylogenetic signal to resolve relationships, and each locus could poten-

tially have a different genealogical history. This, of course, can be an asset as well as a liability by providing replicate estimates from which the organismal phylogeny can be reconstructed. Moreover, a phylogeny derived from multiple loci may reveal near-terminal cladogenic events that could be missed with less data.

The pattern of decreasing diversification rate in *Sphagnum* may be real, and may be an example of density-dependent cladogenesis (Nee et al., 1992). A decreasing rate of diversification was inferred for birds by Nee et al. (1992), based on the phylogeny first published by Sibley and Ahlquist (1990) from DNA-DNA hybridization data. Nee et al. (1992) interpreted the pattern in terms of density-dependent diversification; birds may have first radiated rapidly into new niches (i.e., the air), but diversification subsequently slowed as niche space became more saturated. The slowdown in diversification could have resulted from a lower speciation rate or a higher extinction rate. Nee et al. (1992) noted that the manner in which the diversification rate changed suggests an initially rapid slowdown followed by a leveling off.

Harvey et al. (1994) noted that a variety of explanatory mechanisms may be consistent with a particular tree shape as described by LTT plots or other approaches. Density-dependent diversification, for example, can yield an LTT that is indistinguishable from that produced by a mass extinction accompanied by background patterns of extinction. The curves do differ subtly in expected shape (see Harvey et al., 1994), but are not easy to distinguish statistically. Mooers and Heard (1997: 46) rightly commented that "the fit for any one model should be seen as a starting point for further research, not as an explanation of the data." Models of constant-rate diversification are like other null hypotheses such as the Hardy-Weinberg equilibrium; departures from the null expectation simply tell us that other processes may be at play and warrant investigation.

Density independence (constant diversification) could not be rejected by Purvis et al. (1995) for any of the major clades of primates, although an increase in diversification has occurred in the Cercopithecidae (Old World monkeys). Pybus and Harvey (2000), however, applied a different statistical approach to the primate data and found that constant-rate diversification could be rejected for the Madagascan Strepsirrhini (true lemurs) in favor of a decreasing rate, although not for the New World Platyrrhini. Pybus and Harvey (2000) posed the guarded hypothesis that one interpretation of this result is that the restricted geographic area of Madagascar has led to density-dependent cladogenesis. Mooers and Heard (1997) showed an LTT plot for cranes (Aves: Gruidae), which they interpreted as evidence for density-dependent diversification, but they were not able to reject constant diversification for *Plethodon* salamanders. A constant rate of diversification also could not be rejected for shrews in the genus (*Sorex*), but could be (strongly) rejected for wood warblers (*Dendroica*) (Pybus & Harvey, 2000).

Our results show that statistical tests implemented in DIVERSI need to be interpreted with caution. Alternative models must be assessed for a sample of trees, not just one randomly selected reconstruction. By applying these tests to 20 trees obtained from Bayesian phylogenetic analyses for *Sphagnum*, we were able to show that model A was favored for only 5% of them, while 95% best fit model B. We could therefore reject model A with confidence. Our simulations further showed that phylogenetic stochasticity necessitates an approach to DIVERSI analyses that incorporates replicate tree samples. DIVERSI favored alternative models (B and C) for a significant number of trees, even when simulations were conducted with nearly complete sampling and generated under a model of constant diversification. The LR tests seemed to provide more conservative statistics than did the AIC (Paradis, 1998b). This may have been due in part to the way we conducted these tests. For purposes of Table 2, model B (or C) was favored only when the LR test indicated a statistically significant departure from model A (the null hypothesis). No such significance tests are available for the AIC; alternative models were favored simply when the AIC was lower for those models. Thus, the criterion for rejection of the null hypothesis was more stringent in our LR tests.

Sphagnum is consistently resolved as an early diverging lineage of mosses (e.g., Beckert et al., 1999; Newton et al., 2000; Cox et al., 2004), which implies a long history for the group, although peatmosses could have been around for a long time before the modern levels of species diversity originated. The genus has a poor fossil record with few reports from the Tertiary, so fossils provide little help in determining the chronology of *Sphagnum* diversification (Miller, 1984). *Sphagnum* gametophyte fragments and spores are recorded from Miocene and Pliocene deposits of Europe and North America, and also from China (Miller, 1984; Lu & Zhang, 1989; Ovenden, 1993). Fewer records exist from the Paleogene. It is possible that *Sphagnum* diversified actively as peatlands expanded (presumably during the late Tertiary as global climate

cooled) and underwent extensive niche diversification. Niche differentiation among boreal *Sphagnum* species has been amply documented (e.g., Vitt et al., 1975; Vitt & Slack, 1975; Andrus et al., 1984), with many species occupying well-defined positions along chemical and hydrological gradients. This is one group in which the possibility of density-dependent diversification makes ecological sense. Tests of diversification processes require additional phylogenetic analyses of clades within the genus, and also targeted ecological investigations conducted within a phylogenetic framework.

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POGONATUM (POLYTRICHALES, BRYOPHYTA) REVISITED[1]

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Abstract

Pogonatum, with over 50 species, is the largest genus of the order Polytrichales with four currently distinguished subgenera. The lack of stomata, a deeply pigmented peristome, and papillose exothecium characterize all species that otherwise vary greatly by their size and overall morphology. According to the recent cladistic analyses of the Polytrichales, *Pogonatum* is among the youngest lineages of the order together with *Polytrichastrum, Polytrichum,* and *Eopolytrichum.* Phylogenetic analyses of *Pogonatum* were conducted using morphology and sequence data from the chloroplast *rps*4 and the *trn*L-F gene regions, plus the nuclear-encoded ITS2. Our analyses included all known species of the genus (16 of them represented only by morphology) plus 18 species from other polytrichalean genera used as outgroups. Phylogenetic trees were constructed with simultaneous parsimony analyses of all sequences, plus morphology. Analyses were performed on a reduced data matrix with ambiguously aligned regions deleted, and using direct optimization using all available data. Our results confirm inclusion of *Pogonatum sinense* and species of *Racelopus*-group (*P. camusii, P. iwatsukii, P. marginatum, P. misimense, P. neo-caledonicum, P. petelotii, P. philippinense, P. piliferum, P. rutteri*) within *Pogonatum.* Our results, based on the combined data, do not lend support for a division of the genus into four lineages (e.g., subgenera), although some of the species groups are similar to those resolved based on morphological characters alone.

Key words: cladistics, direct optimization, moss, phylogeny, *Pogonatum,* Polytrichaceae.

The number of currently accepted genera in the Polytrichales is 19, and they accommodate about 200 species (Hyvönen et al., 1998). One affined genus (and its sole species), *Eopolytrichum antiquum* Konopka, Herendeen, Merrill & Crane, is known only as a fossil from the late Cretaceous (Konopka et al., 1997). Seven of the remaining genera are monotypic, and all the others, with exception of *Polytrichum* Hedw. (ca. 30 species.) and *Pogonatum* P. Beauv. (ca. 50 species), are fairly small. Polytrichales are typically pioneer plants of open, sometimes even dry, habitats. They are largely absent from extremely arid regions, however, and the group exhibits its greatest diversity in areas with humid or moist subtropical and tropical climates (e.g., Hyvönen, 1986, 1989; Gradstein et al., 2001). Polytrichales are probably among the first lineages that diverged from the common ancestor to all mosses (e.g., Mishler & Churchill, 1984; Newton et al., 2000). Earlier cladistic analyses of the group are based on morphology (Hyvönen, 1989; Forrest, 1995); more recent ones also used sequences from the chloroplast genic regions *rbc*L, *rps*4, *trn*L-*trn*F, ribosomal RNA gene18S, and mitochondrial *nad*5 (Hyvönen et al., 1998, 2004). According to these recent cladistic analyses, *Pogonatum* is among the youngest lineages of the order together with *Polytrichastrum* G. L. Sm., *Polytrichum,* and *Eopolytrichum.*

The genus *Pogonatum,* originally with 15 species, was separated from the complex genus *Polytrichum* by Paulisot de Beauvois (1804). The genus was gradually broadened by several authors, especially Müller (1848), Dozy and Molkenboer (1854–1870), and Mitten (1859, 1869); now it is the largest genus of the order Polytrichales.

The lack of stomata, a deeply pigmented peristome, and mammillose exothecium characterize all species that otherwise vary greatly by their size and overall morphology. Species of *Pogonatum* are epigeic plants confined to more or less disturbed and open habitats. Some species, such as Southeast Asian *P. neesii,* may even be called weeds. It is an aggressive and successful colonizer of open habitats such as roadsides. Consequently, most species of the genus can be assumed to benefit from human disturbance (Fagerstén, 1977).

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Hyvönen (1989) conducted a manual Hennigian analysis based solely on morphology, proposing a division of the genus into four subgenera: *Alienum* Hyvönen, *Dendroidea* (Schimp.) Hyvönen, *Catharinella* (Müll. Hal.) Hyvönen, and *Pogonatum*. In this study we make phylogenetic inferences based on morphology and sequence data from the chloroplast gene *rps*4 and the *trn*L-*trn*F gene region and nuclear-encoded ITS2. These genic regions were chosen based on the results obtained in our former studies of the whole family (Hyvönen et al., 1998, 2004) plus studies of other moss families (e.g., Huttunen & Ignatov, 2004; Quandt et al., 2004). The three gene regions have proved to be variable at and below genus and species level. Our analyses included all species of the genus *Pogonatum* (16 of them represented only by morphology) plus 18 species from other polytrichalean genera used as outgroups. The choice of the outgroup is based on previous analysis of the whole order. In our earlier study, the genus *Pogonatum* was not resolved as a monophyletic lineage but rather as a paraphyletic group grading into *Polytrichum* (Hyvönen et al., 2004). Here we aim to resolve the phylogeny of the most diverse and species-rich apical clade within the Polytrichales.

Materials and Methods

Our morphological matrix includes 47 characters, and its compilation is based on our earlier analyses of the genus (Hyvönen, 1989) and the whole order (Hyvönen et al., 1998, 2004), plus separate, partly unpublished studies of individual species. This data set is based on an extensive study of specimens from several herbaria during 20 years and includes also the actual DNA voucher specimens. The data matrix and list of characters can be found in Appendices 1 and 2. All species and subspecies included in *Pogonatum* by Hyvönen (1989) and *P. sinense*, a species transferred to *Pogonatum* by Hyvönen and Wu (1993), are included in this analysis. We consider *P. subfuscatum* Broth., a poorly known Chinese species, to belong to *P. contortum*, and thus it is not included. Disagreement also exists on the number of species in Central America. Smith Merrill (1994) recognized 10 species compared to 6 accepted by Hyvönen (1989), with the latter treatment followed here. It should be noted, however, that a separate study with wider population-level sampling is warranted for these species.

DNA was isolated from 58 specimens of *Pogonatum* (representing 56 taxa) and 18 outgroup specimens. The material was either fresh and silica-dried or originated from the herbarium specimens (see Table 1). We were not able to get sequences of all three loci for all specimens (see Table 1) despite repeated trials. *Pogonatum pensilvanicum* is represented by three terminals for this reason; collections from Brazil (JH6393) and the United States, from Alabama (BG5266) and North Carolina (JWH1843), were included in the analyses (see Table 1). Sequence-level data is totally lacking for 18 species (or subspecies) of *Pogonatum*, plus naturally for the fossil *Eopolytrichum antiquum* that is included in the outgroup.

Three genic regions were chosen for the study: (1) the nuclear ribosomal DNA (rDNA) ITS2, (2) plastid small ribosomal protein 4 (*rps*4), and (3) partial region of the genes for tRNA-leucine and tRNA-phenylalanine, hereafter as *trn*L, including one intron and intergenic spacer encoded by the chloroplast genome.

Two different methods were used to extract DNA (Hyvönen et al., 1998), using DNeasy Plant Mini Kit for DNA isolation from plant tissue (QIAGEN, Valencia, California, U.S.A.).

ITS2, *rps*4, and *trn*L sequences were amplified by the polymerase chain reaction (PCR). PCR procedures for *rps*4 and *trn*L are given in Hyvönen et al. (1998). The procedure for ITS2 followed an initial denaturation 94°C for 10 min. to activate *AmpliTaq* Gold DNA polymerase (Perkin-Elmer, Norwalk, Connecticut, U.S.A.), then 35 cycles of denaturation (30 sec. at 95°C), annealing (1 min. at 60°C), and extension (2 min. at 72°C). Primers for ITS2 were ITS3 (forward) and ITS4 (reverse) (White et al., 1990).

PCR and cycle sequencing were done in a Perkin-Elmer 9700 GeneAmp PCR system. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Sequencing reactions were prepared using the BigDye Terminator Cycle Sequencing Reaction Kit v. 2.0 (PE Applied Biosystems, Foster City, California, U.S.A.) and run on the ABI PRISM 377 DNA sequencing sequencer (Perkin-Elmer). Complementary strands were combined using programs of the Lasergene package (DNASTAR, Inc., Madison, Wisconsin, U.S.A.). All sequences were compared to GenBank, EMBL, and DDBJ databases using BLAST for detection of mistakenly amplified amplicons and, especially in the case of ITS2, to avoid fungal sequences.

Sequence alignment was performed initially with the program ClustalX (Jeanmoguin et al., 1998) with default parameters, and sequences were subsequently refined manually in order to maximize positional homology of nucleotides between differ-

ent terminals. For ITS2, this resulted in a matrix of 661 nucleotides and for *trn*L a total length of 665 nucleotides (the original alignments are available from the authors upon request).

The chloroplast gene *rps*4 did not pose alignment problems (the AT-rich indel at the 3′ was not included in the analysis). The *trn*L gene regions, as well as the nuclear spacer ITS2, however, show considerable length variation and therefore alignment is problematic. There are basically two ways to treat ambiguous alignments of sequences. It has been proposed that all such regions should be excluded from the analyses, a procedure that leads to ignoring part of the data. The logic behind this is, however, that there are portions of the sequences for which we can never be sure (at least with the current technology) that we have found the optimal alignment (i.e, that aligned characters are homologous). If we adopt this approach for the current material, we have to ignore most of the ITS2 region and also a large stretches of the *trn*L sequences. Of the ITS2 only the first 111 nucleotides were aligned unambiguously, but this region was so homogeneous that it included only one parsimony informative site. Of the aligned *trn*L matrix with the length of 665 nucleotides, unambiguous alignment was obtained only for the positions 1–129, 150–194, 345–476, 544–590, and 608–665. Altogether the size of the whole matrix was 76 × 1142 cells for all four partitions. Of the total 1142 characters, 203 were informative and the percentage of informative sites in different partitions is given in Table 2. This data matrix has been submitted to TreeBASE (⟨http://www.treebase.org⟩). For the parsimony analyses we used the program Nona (Goloboff, 1993) in conjunction with a Winclada shell (Nixon, 2002). Jackknife support values (Farris et al., 1996) for the nodes were calculated using the program Xac (Farris, 1997).

For the above mentioned analyses we used only part of the available data. Whether sequences showing length variation (i.e., those where assumptions about homology can be challenged) can or should be used in phylogenetic reconstruction is a matter of disagreement. It is quite obvious that these regions, in the same way as reduction and emergence of certain morphological features, can provide valuable information about phylogeny. But how to get hold of this information at sequence level when we have only four (five; A, C, G, T, or a gap) character states that are identical for each character? Homology statements are obviously much more difficult to make than with morphology. It is clear that fast algorithms, for example, those implemented in Clustal and similar programs, do not evaluate alternative alignments; or if this is done, it is performed only to an extremely small extent as compared to all possible alternatives (Slowinsky, 1998). Different solutions to the problem of ambiguous alignment have been proposed (e.g., Lutzoni et al., 2000; Simmons & Ochoterena, 2000), but either they are not implemented in the programs or the programs where they are included are able to handle only a very limited amount of information. These restrictions do not, however, apply to direct (DO, Wheeler, 1996) and fixed states (FSO, Wheeler, 1999) optimizations. Both of these approaches are implemented in the program POY (Wheeler & Gladstein, 2003), and the limit for the number of taxa or length of the sequences that the program is able to process has not yet been reached. This approach has already been used in many large scale phylogenetic analyses (e.g., Giribet et al., 2001). It does not require manipulation of the primary data before the actual analysis (alignment and phylogenetic analyses are done simultaneously). So, there is no need for the special repository for the analyzed matrices, and it is enough to submit sequences used to GenBank. Ideally intact contiguous sequences can be used, but because the alignment is a computationally demanding task, it is advised to cut sequences into smaller fragments to speed up the procedure. When contiguous sequences are cut, assumptions about homology between different sequences are restricted to smaller areas, which leads to large reductions in computational effort. This should, however, be done with great caution in order to avoid unwarranted and premature assumptions about non-homology. It is advisable to cut sequences within regions that are identical in all terminals, i.e., primer areas or other such highly conserved regions. We did this based on preliminary alignment obtained with ClustalX for ITS2 and *trn*L. We were able to cut ITS2 in two and *trn*L in seven fragments. In the ITS2, the first 111 nucleotides of the Clustal alignment included only one informative position, and it was included in the analysis as a single separate character together with the morphological matrix. Direct optimization was used for the rest of the ITS2 region.

We performed four analyses with POY. All these analyses were made with the regions included in the Nona analyses as prealigned and the problematic regions submitted for direct optimization. Three analyses included 76 terminals while the fourth one included only one composite terminal instead of three for *P. pensilvanicum* (see discussion below). These analyses were performed using the IBMSC parallel supercomputer, located at CSC–

Table 1. Vouchers for taxa sampled in analysis, followed by GenBank accession numbers for DNA sequences. All specimens in H if not stated otherwise.

Taxon	Collection reference	GenBank accession No.		
		*rps*4	*trn*L	ITS2
Ingroup				
Pogonatum aloides (Hedw.) P. Beauv.	Sweden. Skåne, *Hyvönen 6486*	AY137689	AF545016	—
P. belangeri (Müll. Hal.) A. Jaeger	Tanzania. Tanga Province, *Laaka 298*	AY396517	AY396499	AY396474
P. brachyphyllum (Michx.) P. Beauv.	U.S.A. Georgia, *Shaw 8304*	AY396515	AY396497	AY396473
P. campylocarpum (Müll. Hal.) Mitt.	Brazil. São Paulo, *Hyvönen 6392*	AY137690	AF545017	AY396455
P. cirratum (Sw.) Brid. ssp. *cirratum*	Taiwan. Taichung, *Hyvönen 4008*	AY137691	AF545018	AY396448
P. cirratum ssp. *fuscatum* (Mitt.) Hyvönen	China. Hunan, *Koponen 49039*	—	AY396494	AY396470
P. cirratum ssp. *macrophyllum* (Dozy & Molk.) Hyvönen	Malaysia. Aug. 1998 *Tan s.n.*	AY396502	AY396478	AY396443
P. comosum (Müll. Hal.) Mitt.	Mexico. Oaxaca, *Norris 77508*	AY396503	AY396481	AY396446
P. contortum (Brid.) Lesq.	Canada. British Columbia, *Hedderson 5803*	AF208425	AF545019	—
P. dentatum (Brid.) Brid.	Finland. Uusimaa, *Hyvönen 6169*	AY137692	AF545020	AY396454
P. fastigiatum Mitt.	Taiwan. Nantou, *Hyvönen 3556*	AY396504	AY396482	AY396447
P. gracilifolium Besch.	Uganda. Rukungiri, *Wigginton 5071a*	AY396507	AY396488	AY396462
P. inflexum (Lindb.) Sande Lac.	Japan. Honshu, *Chishiki 1865*	—	AY396486	AY396459
P. japonicum Sull. & Lesq.	Japan. Honshu, *Nishimura 10601*	AY137693	AF545021	AY396463
P. microstomum (Schwägr.) Brid.	Taiwan. Taichung, *Hyvönen 4087*	AY137694	AF545022	AY396450
P. nanum (Hedw.) P. Beauv.	Sweden. Skåne, *Hyvönen 6484*	AY396506	AY396484	AY396456
P. neesii (Müll. Hal.) Dozy	Taiwan. Taichung, *Hyvönen 4021*	AY137695	AF545023	AY396449
P. neglectum (Hampe) A. Jaeger	Colombia. Antioquia, *Churchill 16370*	AY396510	AY396491	AY396466
P. neo-caledonicum Besch.	New Caledonia. *Norris 93319*	AY396513	AY396495	AY396471
P. nipponicum Nog. & Osada	Japan. Honshu, *Hayashi 7038*	AY137696	AF545024	AY396460
P. nudiusculum Mitt.	Taiwan. Taichung, *Hyvönen 4153*	AY396505	—	AY396451
P. pensilvanicum (Hedw.) P. Beauv.	Brazil. São Paulo, *Hyvönen 6393*	AY137697	AF545025	—
P. pensilvanicum	U.S.A. Alabama, *Goffinet 5266*	—	AY396477	AY396442
P. pensilvanicum	U.S.A. North Carolina, *Horn 1843*	—	AY396498	—
P. pergranulatum P. C. Chen	China. Sichuan, *Allen 6501*	AY396514	AY396496	—
P. perichaetiale (Mont.) A. Jaeger	Bolivia. Inquisivi, *Lewis 87019* (MO)	—	AY396487	AY396461
P. piliferum (Dozy & Molk.) Touw	Papua New Guinea, *Koponen 36036*	AY396512	—	AY396469
P. procerum (Lindb.) Schimp.	Honduras, La Paz, *Liesner 26480*	AY396508	AY396489	AY396464
P. proliferum (Griff.) Mitt.	Uganda. Buhoma, *Porley 652a*	AY396509	AY396490	AY396465
P. rufisetum Mitt.	Bhutan. Thimphu, *Long 10801*	—	AY396479	AY396444
P. semipellucidum (Hampe) Mitt.	Colombia. Antioquia, *Churchill 18679*	AY396511	AY396492	AY396467
P. sinense (Broth.) Hyvönen & P. C. Wu	Bhutan. Mongar, *Long 8696*	—	AY396480	AY396445
P. spinulosum Mitt.	Japan. Honshu, *Chishiki 1862*	AY137698	AF545026	AY396457

Table 1. Continued.

Taxon	Collection reference	GenBank accession No.		
		*rps*4	*trn*L	ITS2
P. subtortile (Müll. Hal.) A. Jaeger	Papua New Guinea, *Koponen 34850*	—	AY396493	AY396468
P. subulatum (Brid.) Brid.	Australia. Queensland, *Hyvönen 6025*	AY137699	AF545027	AY396453
P. tahitense Schimp.	U.S.A. Hawaii, *Hyvönen 4904*	—	AY396483	AY396452
P. tortile (Sw.) Brid.	Puerto Rico, *Goffinet 5067*	AY396501	AY396476	AY396441
P. tubulosum Dixon	Papua New Guinea. *Hoffmann 89-741*	—	AY396485	AY396458
P. urnigerum (Hedw.) P. Beauv.	Finland. Uusimaa, *Hyvönen 6173*	AF208426	AF545028	AY396472
P. usambaricum (Broth.) Paris	Tanzania. Tanga Province, *Laaka 3235*	AY396518	AY396500	AY396475
Outgroup				
Atrichum androgynum (Müll. Hal.) A. Jaeger	Brazil. São Paulo, *Hyvönen 6387*	—	AF544999	AY396432
A. angustatum (Brid.) Bruch & Schimp.	U.S.A. Louisiana, *Hedderson 10393* (RNG)	AF208417	AF545000	AY396430
A. oerstedianum (Müll. Hal.) Mitt.	Mexico. Veracruz, *Hyvönen 6504*	AY137680	AF545001	AY396431
A. undulatum (Hedw.) P. Beauv.	Finland. Uusimaa, *Hyvönen 6170*	AY137681	AF545002	AY396433
Meiotrichum lyallii (Mitt.) G. L. Sm.	U.S.A. Colorado. *Weber WWB36612*	AF208423	AF545011	AY396426
Oligotrichum hercynicum (Hedw.) Lam. & DC.	Finland. Uusimaa, 25 July 1998, *Enroth s.n.*	AY137688	AF545014	AY396427
O. parallelum (Mitt.) Kindb.	Canada. British Columbia, *Hedderson 10043* (RNG)	AF208424	AF545015	AY396428
Polytrichastrum alpinum (Hedw.) G. L. Sm.	Finland. Etelä-Häme, *Hyvönen 6204*	AY137701	AF545031	—
P. formosum (Hedw.) G. L. Sm.	Finland. Uusimaa, *Hyvönen 6197*	AY137702	AF545032	AY396435
P. longisetum (Sw. ex Brid.) G. L. Sm.	Finland. Varsinais-Suomi, *Hyvönen 6506*	AY137703	AF545033	—
Polytrichum brachymitrium Müll. Hall.	Brazil. Minas Gerais, *Hyvönen 6230*	AY137704	AF545034	AY396436
P. commune Hedw.	Finland. Uusimaa, *Hyvönen 6168*	AF208428	AF545035	AY396437
P. juniperinum Hedw.	Finland. Uusimaa, *Hyvönen 6193*	AY137705	AF545036	AY396438
P. piliferum Hedw.	Finland. Uusimaa, *Hyvönen 6205*	AY137706	AF545037	AY396439
P. subpilosum P. Beauv.	Malawi. Mulanji, *Wigginton M1397a*	AY137707	AF545038	AY396440
Psilopilum laevigatum (Wahlenb.) Lindb.	Canada. Ellesmere Island. *Hedderson 5938* (RNG)	AF208429	AF545039	AY396434
Steereobryon subulirostrum (Schimp.) G. L. Sm.	Mexico. Veracruz, *Hedderson 12898*	AY137708	AF545040	AY396429

Table 2. Data matrix with the percentage of parsimony informative characters in each partition used in the analyses of static alignments.

Matrix	Characters	Informative	(%)
ITS2	111	1	1
*rps*4	573	120	21
*trn*L-F	411	35	9
Morphology	47	47	100
Combined total	1142	203	18

Scientific Computing Ltd., Espoo, Finland. This is an IBM eServer Cluster 1600 system, constructed of 16 pSeries 690 nodes (each equipped with 32 POWER4 processors) and an SP Switch2 (Colony). Sixteen processors of one node were used for our analyses. Command lines used can be found in Appendix 3.

Results

Simultaneous parsimony analyses of the combined matrix from three gene regions, plus morphology using Nona within Winclada shell, were performed with the following settings: hold * (holding all trees that memory allows, in current settings with Winclada this is 10,000), mult*100 (search replicated 100 times), and hold/2 (keeping 2 starting trees for each replication). Using the multiple tree-bisection reconnection algorithm (mult*max*) resulted in 225 equally parsimonious trees (EPTs) of 862 steps (CI 0.52, RI 0.67). Only 1% of the initial searches found this minimum length tree, so it was warranted to make a larger analysis. This was performed with mult*1000 and hold/10, and this time 7113 EPTs were found. Here, the resolution was lost quite dramatically, and because only 28 out of 1000 replicates found shortest trees, we performed also ratchet (Nixon, 1999) searches. These were performed with 1000 iterations per replicate, two trees held per iteration, and reweighting 15%, 20%, and 25% of the total number of characters. Treatment of ambiguous optimization of characters was set as amb = poly-, which means that branches were collapsed only if ancestor and descendant have the same state for all characters. This led to finding 372 (15% reweighting), 469 (20%), and 451 (25%) EPTs with the length of 861 steps. When the results of all these searches were pooled, we had 8556 EPTs. The strict consensus of these trees is illustrated in Figure 1.

Two of the 8556 EPTs were optimal based on morphology, and these trees are illustrated in Figure 2 with unambiguous character state changes mapped on the trees. For discussion of character evolution and clades found on these two trees, see below.

In the first analysis with POY using direct optimization, 19.5 million (M) trees and over 630 M alignments were examined. This analysis resulted in four equally parsimonious alignments and trees. The second analysis with more thorough heuristics (52.3 M trees, number of performed alignments not recorded because of a mistake) did not find these optimal trees. The third analysis with further expanded heuristics (85.6 M trees and 935 M alignments) resulted in one optimal tree that was five steps shorter than the four trees found in the first analysis. This tree is illustrated in Figure 3.

Discussion

The clades present in the strict consensus of the 8556 trees (obtained from the analyses based on prealigned sequences) are mostly composed of the species that one would expect to be closely related based on earlier analysis (Hyvönen, 1989) and morphology. The relationships of the outgroups are unaltered from those obtained in the study of the whole family (Hyvönen et al., 2004) with one exception: *Psilopilum* Brid. appears to be within the clade formed by *Atrichum* P. Beauv., *Oligotrichum* Lam. & DC., and *Steereobryon* G. L. Sm., and not sister to the apical clade formed by *Eopolytrichum*, *Pogonatum*, *Polytrichastrum*, and *Polytrichum*. *Pogonatum* is monophyletic but internal resolution is very poor with only small clades of few species present (but without jackknife support). The largest clade is the one formed by nine small species of the *Racelopus* group (Touw, 1986). It should also be noted that *Polytrichastrum alpinum*, a species already treated as a species of *Pogonatum* by some authors (e.g., Osada, 1965; Crum & Anderson, 1981), is within this genus. The present results confirm some conclusions made in earlier studies. Touw (1986) synonymized *Pseudoracelopus* Broth., *Racelopodopsis* Thér., and *Racelopus* Dozy & Molk. with *Pogonatum*, and our results support this. Species of Touw's *Racelopus* group appear again as a monophyletic entity but firmly within *Pogonatum*. Similarly, inclusion of *Microdendron sinense* Broth. within *Pogonatum*, as proposed by Hyvönen and Wu, (1993) is confirmed here.

The small clades present in the strict consensus tree (Fig. 1) are compatible with groupings presented in Hyvönen (1989), with one exception—the pair formed by *Pogonatum tahitense* and *P. campylocarpum*. These two were included in different subgenera in 1989. However, when we examine the two trees that are optimal according to the mor-

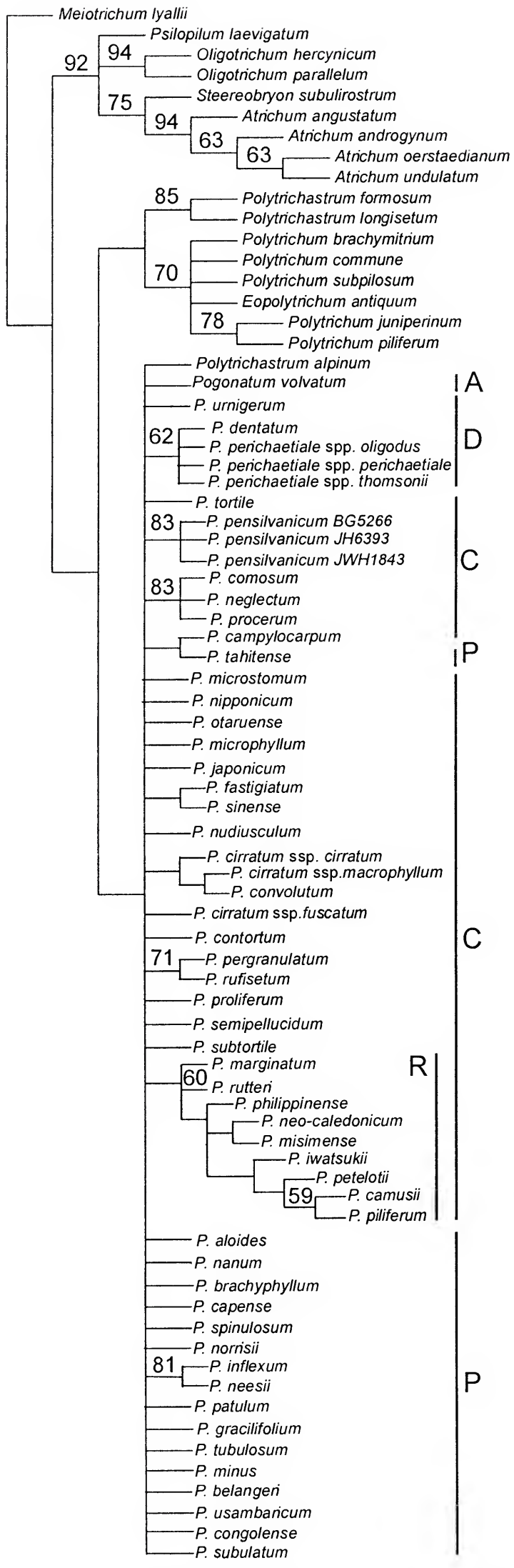

Figure 1. The strict consensus tree based on 8556 equally parsimonious trees (L 862 steps) obtained with simultaneous analysis of three genic regions (ITS2, *rps*4, *trn*L-F), plus morphology. Jackknife support values exceeding 50% are marked by the nodes. Division of the genus *Pogonatum* according to Hyvönen (1989) is shown with the letters referring to the species of each subgenera with A for the subgenus *Alienum*, D for *Dendroidea*, C for *Catharinella*, and P for *Pogonatum*. Additionally, the Racelopus group (Touw, 1986) has been marked with R. Specimen numbers shown reflect collector initials and number (Table 1).

phology, it is evident that the present results do not support the subgenera distinguished earlier. Instead of *P. volvatum* (Müll. Hal.) Paris, the basalmost species is *P. microstomum*. All other species forming the basal grade are those of the subgenus *Dendroidea* and *Alienum*, except *P. japonicum*. All these species are, however, plants with fairly thick cell walls in the leaves and many of their features (e.g., large size, dentate leaf margins) are characters that are typical for species of *Polytrichastrum* and *Polytrichum*. The species formerly assigned to the subgenera *Catharinella* and *Pogonatum* (with the exceptions given above) are all included in the large apical clade (Fig. 2). The internal divisions of this clade do not correspond to former subgenera; however, the division into these two subgenera can still be retained, with somewhat altered composition. Based on the results of the present study the smaller group, subgenus *Pogonatum*, around *P. aloides*, is formed of 10 species, and the larger group, subgenus *Catharinella*, is composed of 36 species (Fig. 2).

In the POY analyses, relationships of the outgroups are altered from that of all earlier analyses (Fig. 3). *Oligotrichum* is the first lineage diverging from the common ancestor after *Meiotrichum* G. L. Sm., and after that it is *Atrichum*, *Psilopilum*, and *Steereobryon* that is a sister clade to *Eopolytrichum*, *Polytrichastrum*, and *Polytrichum*, not *Pogonatum* as proposed by our earlier analysis of the whole family (Hyvönen et al., 2004). However, *Pogonatum japonicum* together with *Pogonatum volvatum* are within the latter clade! *Pogonatum microstomum* appears to be the basalmost member of *Pogonatum*, together with *Polytrichastrum alpinum* and *Pogonatum urnigerum*. The position of *P. japonicum* and *P. volvatum* is surprising. For the latter species we did not have any sequence data, only morphology, which certainly resembles more that of *Polytrichastrum* or *Polytrichum* (or *Pogonatum japonicum*) than other species of *Pogonatum* as noted already by Smith (1975) and Hyvönen (1989). Both species, however, have a typical *Pogonatum* capsule—deeply pigmented peristome with 32 compound teeth and mammillose exothecium that lack stomata. Are these two plants possibly intermediates resulting from ancient hybridization between

←

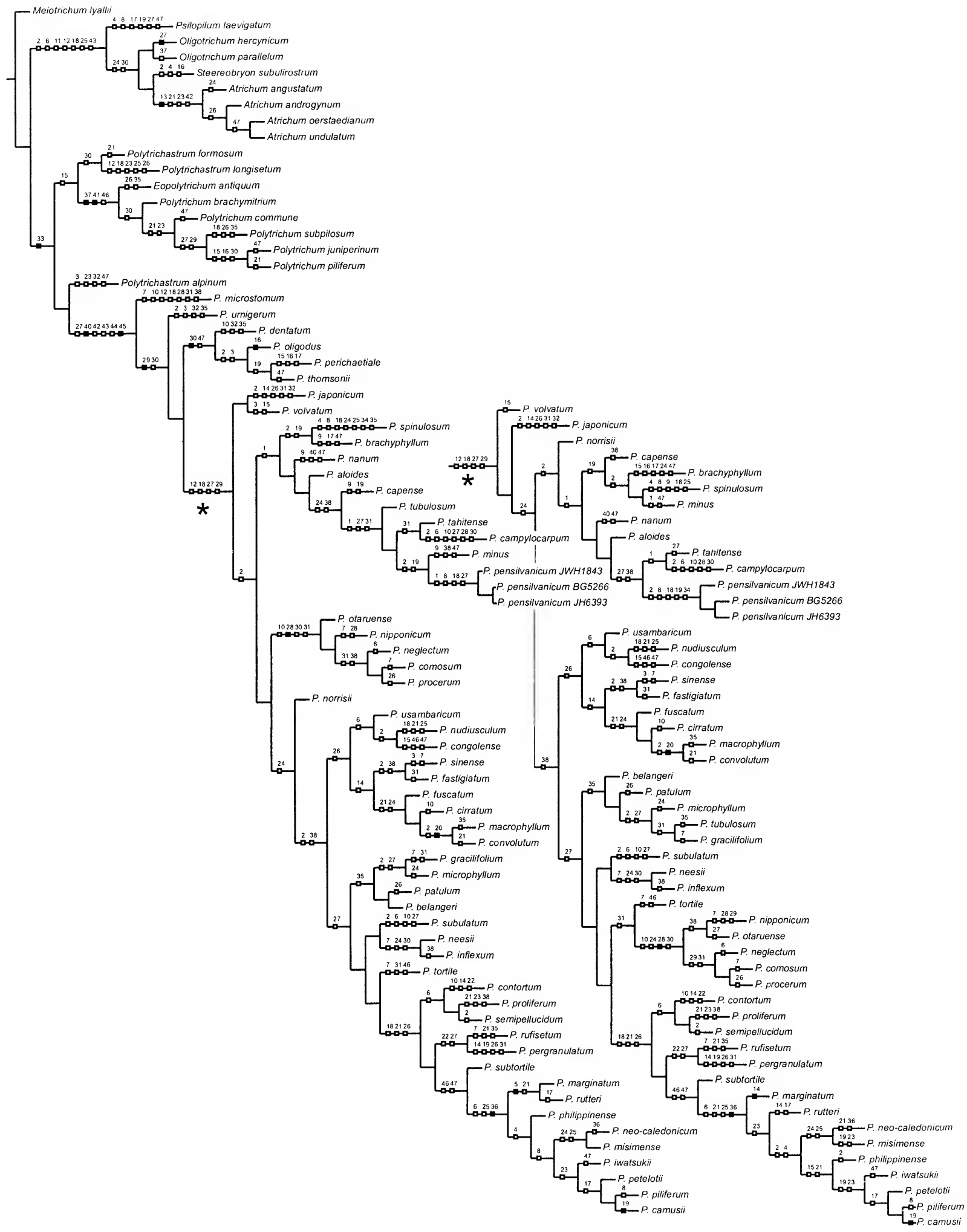

Figure 2. Two equally parsimonious trees (out of 8556) that are optimal based on morphology. Unambiguous character state changes are marked on the nodes and empty nodes are those with support only from sequence-level data. White squares indicate homoplasy (discontinuous states). Asterisks (*) indicate where the two topologies start to differ.

two lineages? Additional sampling from both chloroplast and nuclear genome is needed to confirm or refute this. As in the analysis based on the static alignment above, the internal division of *Pogonatum* does not correspond to former subgenera, but this division can be retained with altered composition.

When we examine morphological characters, it

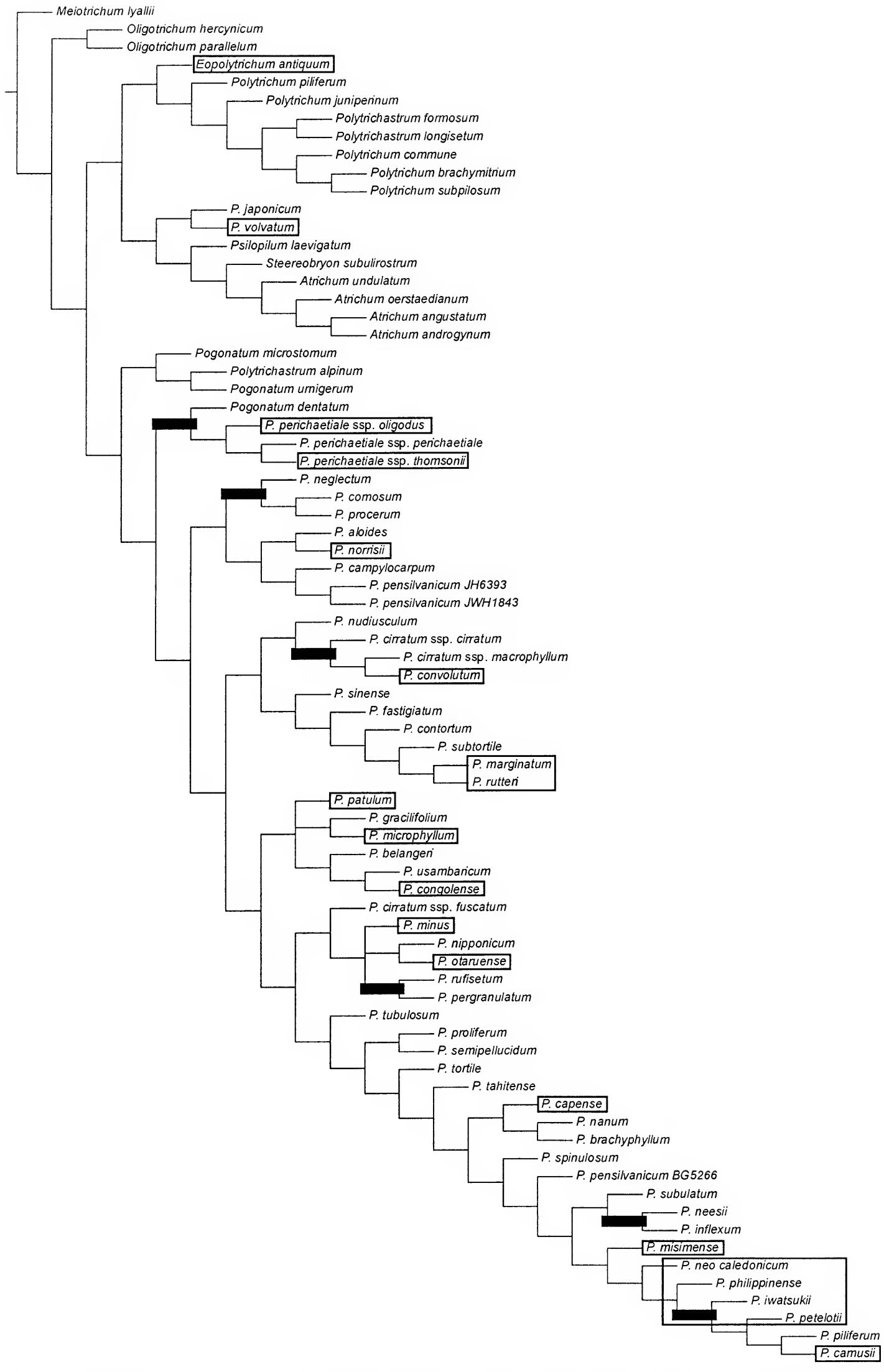

Figure 3. The single parsimonious tree obtained based on direct optimization. Enclosed terminal names indicate those species that were included in the analysis based only on morphology. Branches emphasized with black bars are those that are present on the strict consensus tree based on 8556 obtained through analysis of static alignment.

seems that none of them are very good indicators of phylogeny, since all of them show considerable homoplasy. However, the level of homoplasy observed does not differ significantly from the values obtained for sequence-level data. The combined simultaneous analysis provided results that would have been unexpected based solely on morphology. In the following we will briefly discuss some of the morphological characters based on their distribution on two trees out of 8556 that are optimal based solely on morphology (Fig. 2).

Many species of *Pogonatum* have reduced leaves (char. 8), and it seems that this reduction has taken place independently for *P. spinulosum*, *P. pensilvanicum*, and a third time for the species of the *Racelopus* group. However, the species in the latter group form a monophyletic entity on the other one of these two trees. The other extreme of the leaf development is exemplified by species like *P. cirratum* ssp. *macrophyllum* and *P. convolutum*. They have a secondary ventral stereid band in their leaves (char. 20) like the large species of *Dawsonia* R. Brown, and it seems that this character has developed in *Pogonatum* only once. In some species not only the blade of the leaves but also the number of adaxial lamellae is clearly reduced or they are totally absent (char. 25). These two characters are not, however, coupled with each other. Species like *P. nudiusculum*, *P. proliferum*, and *P. semipellucidum* have otherwise well-developed leaves, but the adaxial lamellae are either restricted to the very central part of the leaf (*P. nudiusculum* and *P. semipellucidum*) or they are present on the ventral side of the very narrow costa only (*P. proliferum*). These three species do not, however, form a monophyletic group. *Pogonatum nudiusculum* is in a clade together with *P. usambaricum* and *P. congolense* Card., and these two species do show fairly wide leaf margins that are devoid of lamellae, although this quantitive difference has not been distinguished as a distinct character state. Such species as *P. spinulosum* and all the species of the *Racelopus* group lack lamellae altogether, or they are very reduced like in *P. neo-caledonicum* and *P. misimense* (Bartr.) Touw. The latter two are, however, nested within the *Racelopus* group (see Fig 1.) and for these two we have to accept a reversal (at least partial) to an earlier character state. Apical cells of lamellae (lamellae marginal cells) are useful characters to distinguish species of *Pogonatum*, and this was considered to be an important taxonomic character by Hyvönen (1989). Apical cell size (compared to other cells of the lamellae) and form, as seen in cross section (chars. 28, 30), were used to distinguish a group of predominantly South American species as a monophyletic group within *Pogonatum* subg. *Catharinella* in Hyvönen (1989). Although all these species greatly resemble each other in these two characters, this is not without homoplasy on current trees. *Pogonatum microstomum* is consistently in a basal position (Figs. 2, 3), and *P. campylocarpum* also seems to belong to another group of species not closely related to other American species with these characteristic apical cells. These species (*Pogonatum comosum*, *P. neglectum*, and *P. procerum*) share a common ancestor with *P. otaruense* and *P. nipponicum* from Japan (Fig. 2).

When we examine the tree based on direct optimization, there is another unexpected result (in addition to the grouping of *P. japonicum* and *P. volvatum*), namely, *P. pensilvanicum* appearing as polyphyletic. Three specimens of *Pogonatum pensilvanicum* are present as a monophyletic group on the strict consensus based on static alignment. These terminals are similar in the morphological characters included, and this strange result is quite obviously an artifact and not due to the actual phylogeny. We did not have complete sequence-level character partitions for any of these three terminals (see Table 1). They are similar in their *trn*L sequences, differing only by the length so that BG5266 (Alabama) is 18 nucleotides (nts) longer than JWH1843 (North Carolina) and 13 nts longer than JH6393 (Brazil). The terminals from Brazil and North Carolina form a clade and group together with *P. campylocarpum*, while the plant from Alabama appears within the apical clade close to *P. spinulosum* and *P. subulatum*, *P. neesii*, and *P. inflexum*. Which one of these two positions should we consider more reliable? In order to test this we performed direct optimization analysis with the same settings as for the whole material, but now reduced to 74 terminals so that we had a composite of *P. pensilvanicum* specimens with chloroplast sequences of JH6393 from Brazil supplemented with ITS2 sequence of BG5266 from Alabama. With this analysis we obtained two parsimonious alignments and trees, and on both trees (not shown) *P. pensilvanicum* appeared within the clade including *P. campylocarpum* and allies. It seems that *P. pensilvanicum* BG5266 may be drawn out of this clade in the original analysis due only to its lack of *rps*4 sequence.

Based on the results of direct optimization and the two trees of the Nona analyses that were optimal and based on morphology, we can draw some conclusions about general trends in the evolution of *Pogonatum*. This is now easier than in former analyses such as that by Hyvönen (1989) because we

now have a robust hypothesis of phylogeny of the whole family (Hyvönen et al., 2004). It appears that the common ancestor of all species of the genus was a fairly large plant with numerous adaxial lamellae on well-developed leaves. In both major clades within the genus, evolution has proceeded towards reduced, and in some cases extremely reduced, structure. It seems that extreme neoteny has been a successful strategy for these plants on almost all continents and in diverse biomes. In future studies it would be interesting to try to estimate the age of these extremely reduced clades and compare them with estimates from other groups of bryophytes growing in similar habitats. At the moment, age estimates for different lineages of bryophytes are still lacking, and different approaches for this kind of age estimation are actively developing (e.g., Sanderson, 2003).

While our results confirm some of the nomenclatural conclusions made in earlier studies (Hyvönen & Wu, 1993; Touw, 1986), we also face some new and complicated challenges. In the tree based on direct optimization, two species of *Pogonatum*, *P. japonicum* and *P. volvatum*, appear clearly outside the genus. Should these two be transferred to another genus? Similarly problematic is the position of *Polytrichastrum alpinum*. It appears nested within *Pogonatum*, but on trees that are optimal based on morphology it can equally well be left out, retaining its generic status. We agree with Wiley (1981) that nomenclature should reflect phylogeny as closely as possible, but at the same time premature alterations should be avoided. This means that we hesitate to create a new genus for *P. japonicum* and *P. volvatum* especially because we did not have sequence data for the latter and because different analyses, including the one based on a larger body of characters but limited taxonomic sampling, do not agree on their position. What seems to be clear, however, is that *Polytrichastrum* as currently delimited is not a monophyletic entity. This pattern has now emerged in several analyses, although it should be noted that the sampling is still far from being comprehensive. If we want to minimize nomenclatural changes and still also present our results as congruent as possible with nomenclature, both *P. formosum* and *P. longisetum* should be assigned to a new genus. If it appears (with extended character sampling) that *Polytrichastrum alpinum* is indeed within *Pogonatum*, this would make *Polytrichastrum* superfluous and available to accommodate both *P. formosum* and *P. longisetum*. This would, however, necessitate a nomenclatural proposal to change the generic type, which is currently *Polytrichastrum alpinum*. Taking all this together, if we move both *Polytrichastrum formosum* and *P. longisetum* back to *Polytrichum*, then the original names *Polytrichum formosum* Hedw. and *P. longisetum* Sw. ex Brid., respectively, may be applied. Whether *Eopolytrichum antiquum* Konopka et al. (Konopka et al., 1997) should be as well included within *Polytrichum* s.l. remains to be seen. This becomes necessary if *Eopolytrichum antiquum* proves to be nested within *Polytrichum* with more thorough analyses. At the moment, however, our results are indecisive as to its proper nomenclatural treatment.

Future studies should be directed toward expanded character sampling of *Pogonatum*. The obvious choice for this would be the chloroplast gene *rbc*L that has already been sampled for a fair number of species (Hyvönen et al., 2004). When the 14 species of *Pogonatum* (with *Polytrichastrum alpinum* included) are compared, their *rbc*L sequences still include 17% informative sites. However, much more effort is needed to get these sequences because *rbc*L is both longer and not as easy to amplify as the two other chloroplast regions used here. Equally important in future analyses would be the inclusion of the few, as yet, unsampled species, especially species like *P. volvatum*, that appear in a basal or in a very unexpected position on current trees. And, first of all, we should fill in the gaps in our current matrix, as not all sequences were obtained for all terminals for the current analyses.

Our analyses, based on direct optimization, show that sequences with length variation do contain valuable information for phylogeny reconstruction in *Pogonatum*. However, it should be acknowledged that with the analyses presented here we have explored only a tiny fraction of possible alignments and trees, and it would not be surprising that more extensive heuristics will reveal better alignments and shorter trees, possibly with much altered topology. While some guidelines can be given about adequacy of heuristic analyses on static alignments after a decade of experience with different search strategies and algorithms, the same is not true (as yet) when we deal with direct optimization. These methods have not yet been extensively used, and therefore much remains to be learned about their behavior and performance. They are continuously developed, and novel; improved approaches that challenge earlier results are presented almost annually (e.g., Grant & D'Haese, 2003; Wheeler, 2003a, 2003b).

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Appendix 1. Morphological characters and character states.

1. Protonema fugacious (0); persistent (1).
2. Size: plants robust > 180 mm (0); large, 50–110 mm (1); small, 10–40 mm (2); depauperate, < 7 mm (3).
3. Branching: stems unbranched (0); branching common (1); dendroid (2).
4. Well defined (polytrichoid) central strand present (0); absent (1).
5. All cauline leaves isophyllous (0); anisophyllous (1).
6. Sheath distinct and broad (0); not differentiated (1).
7. Rhizoids on leaves present (0); absent (1).
8. Leaf blade normal, longer than sheath, ratio > 1.5 (0); reduced, ratio < 1 (1); essentially absent (2).
9. Leaf apex acute, essentially plane (0); distinctly cucullate (1).
10. Sheath margins entire or crenate by bulging cells (0); entire or serrate (1); uniformly serrate (2).
11. Hyaline leaf margins present (0); absent (1).
12. Hinge tissue between blade and sheath absent (0); present (1).
13. Leaf border absent (0); *Atrichum*-type (1).
14. Blade margins unistratose (0); bistratose (1); multistratose (2).
15. Blade margins dentate (toothed) (0); serrate (1); crenate by bulging cells or entire (2).
16. Dentation or serration essentially along whole blade (0); serrate portion less than 1/3 of the blade length or entire (1); uniformly entire (2).
17. Dorsal side of costa serrate (0); entire (1).
18. Dorsal stereid band almost as wide as blade (0); narrowed (1); present only in costa that is restricted only to central part of leaf (2).
19. Stereid bands present (0); only dorsal band present (1); absent (2).
20. Number of stereid bands 2 (0); 3 with secondary ventral stereid band present (1).
21. Cell walls of stereid bands moderately incrassate (0); dorsal band with pale incrassate walls (1); both bands with incrassate walls (2).
22. Cell walls of central cells thin (0); distinctly incrassate (1).
23. Cell walls of dorsal cells of blade extremely incrassate with cell-lumen small and rounded (0); firm, evenly incrassate walls with cell lumen subquadrate (1); thin or firm walls, small thickenings at corners (2); very thin, cells collenchymatous (3).
24. Outer wall of dorsal cells of costa incrassate, thicker than transverse cell walls (0); thin to firm, similar to transverse cell walls (1).
25. Ventral lamellae covering practically whole lamina (0); restricted to central part (1); present only on ventral side of narrow costa (2); lamellae absent (3).
26. Height of lamellae > 4 cells (0); 3 to 4 cells (1); 1 to 3 cells (2).
27. Lamellae (apical) margins straight or indistinctly crenate as seen in side view (0); distinctly crenate (1); irregularly crenate (2); serrate (3).
28. Size of LMC (lamellae marginal cell) lumen similar to lower cells of lamellae (0); elongated as seen in side view (1); higher than lower cells (2).
29. LMC cell walls undifferentiated (0); incrassate (1); only outer wall incrassate (2); outer wall notched (3).
30. Form of LMCs (as seen in cross section) flattened (0); undifferentiated (1); ovoid to bottle-shaped (2); retuse (3).
31. LMCs solitary (0); mostly geminate (1); geminate apicals present (2).
32. LMC apical cell walls smooth or very finely papillose (0); coarsely papillose (1).
33. Calyptra with ample rhizoids (0); rhizoids sparse (1).
34. Perigonia elongated (0); short and ellipsoid (1).
35. Paraphyses (among perigonia) uniseriate (0); at least some paraphyses with club-shaped multiseriate apical portion (1).
36. Seta smooth (0); scabrose (1).
37. Apophysis tapering (0); contracted (1); discoid (2).
38. Angles of capsule absent (0); 4 or very rarely more (1); capsules with 6 to 8 folds (2).
39. Capsule angle form blunt (0); sharp, knife-edged (1); ribbed (2).
40. Exothecium smooth or mammillose (0); papillose (1).
41. Exothecial pitting none (0); thin spots (1); pitted (2).
42. Stomata on capsules present (0); absent (1).
43. Peristome teeth 64 (0); 32 (1); 16 (2).
44. Tooth structure simple (0); compound sinus broad (1); compound, sinus narrow (2).
45. Peristome pigmentation pale (0); intensely colored (1).
46. Spore surface ornamentation granular (0); echinoid (1).
47. Spores large, diameter mostly > 18 μm (0); medium-sized, diameter mostly 8.5–15.5 μm (1); small, diameter mostly < 8.3 μm (2).

Appendix 2. Character matrix of morphology. Different character states are coded with (0, 1, 2, and 3) that refer to Appendix 1. Unknown information marked with (?) and inapplicable characters with (–). Specimen numbers shown reflect collector initials and number (see Table 1).

	000000000111111111122222222223333333333444444444 123456789012345678901234567890123456789012345678901234567
Meiotrichum lyallii	011000100001001000000010000022011000111010000001
Oligotrichum hercynicum	020001101010001002000011203001001010000-00012001
O. parallelum	020001100010001002000011200001001??010-00011001
Steereobryon subulirostrum	030101101010002202000011200001001??000-000100?1
Atrichum androgynum	020001100010100002001021220001001??000-00110001
A. angustatum	020001100010100002001020200001001000000-00110001
A. oerstaedianum	020001100010100002001021220001001000000-00110000
A. undulatum	020001100010100002001021220001001000000-00110000
Psilopilum laevigatum	020101111010002012 1-00102020020010100 0-00010000
Polytrichastrum alpinum	011000100001001000000000000022010000100000000000
P. formosum	010000100001000000002010000001000000110000000001
P. longisetum	010000100000000002000020110001000000010000000001
Eopolytrichum antiquum	????00?0?0?10????00000??01?00200?01?20-0200??1?
Polytrichum brachymitrium	010000100001000000000010000003000000211020000?1
P. commune	000000100001000000002000000003000000211020000 12
P. juniperinum	010000100001002200002000001032000000211020000 12
P. piliferum	020000100001002200001000001032000000211020000 11
P. subpilosum	000000100001000001002000011033000010211020000?1
Pogonatum aloides	120000100010001001000010000001000000000-10112101
P. belangeri	010000100010001001000011001001000010022101121 01
P. brachyphyllum	130000101010002211100010000001000000000-10112100
P. campylocarpum	010001100110001001000011001202100000022101121 01
P. camusii	1301011100?00120--2---213-------000102210112112
P. capense	120000101010001001 1-00110000010000002210112101
P. cirratum ssp. *cirratum*	010000100110011001002000020001200000022101121 01
P. cirratum ssp. *macrophyllum*	000000100010011001012010020001200010022101121 01
P. cirratum ssp. *fuscatum*	010000100010011001002000020001000000022101121 01
P. comosum	010000000110001001000010001132100000022101121 01
P. congolense	020001100010002001000011020001000000022101121 12
P. contortum	010001100110011002001111021001000000022101121 01
P. convolutum	000000100010011001011010020001000000022101121 01
P. dentatum	010000100111001000000100010100100 1000-10112100
P. fastigiatum	000000100110011001000011020001100000000-10112101
P. gracilifolium	020000000010001001000011002001200010022101121 01
P. inflexum	010000000010001001000010001003000000000-10112101
P. iwatsukii	1301011100?00020021-10213-------0??102210112111
P. japonicum	000000100010011001000010010001110000000-10112101
P. marginatum	?100111000?00210020000113-------000102210112112
P. microphyllum	020000100010001001000010002001000010022101121 01
P. microstomum	010000000110001001000010001222100000022101121 01
P. minus	030000101 0?00010011?00110020012 00??000-10112 1?2
P. misimense	1301011100?00010021-0010221--100000102210112112
P. nanum	120000101010001001000010000001000000000-00112100
P. neesii	010000000010001001000010001003000000022101121 01
P. neglectum	020001100110001001000010001132100000022101121 01
P. neo-caledonicum	1301011100?000100200203022100100000022101121 12
P. nipponicum	020000000110001001000010001222200000000-10112101
P. norrisii	020000100010001001000011000001000??000-10112101
P. nudiusculum	020001100010001002002011120001000000022101121 01
P. otaruense	020000100110001001000010002102200000000-10112101
P. patulum	010000100010001001000011011001000010022101121 01
P. petelotii	1301011100?00020121-10213-------0??10221011 2?2
P. pensilvanicum BG5266	13000-1100100010021-00110010012001000221011210 1

Appendix 2. Continued.

	000000000111111111122222222223333333333444444444 123456789012345678901234567890123456789012345678901234567
P. pensilvanicum JH6393	13000-1100100010021-00110010012001000221011210 1
P. pensilvanicum JWH1843	13000-1100100010021-00110010012001000221011210 1
P. pergranulatum	0100001000?00110021-11110020012000000??101121??
P. perichaetiale ssp. *perichaetiale*	02100010001100221010001000101000000000-10122100
P. perichaetiale ssp. *oligodus*	01200010001100110000001000101000000000-10122100
P. perichaetiale ssp. *thomsonii*	02100010001100100010001000101000000000-10112101
P. philippinense	12010110001000200200103 13-------000102210112112
P. piliferum	1301011200?-----121-1--13-------000102210112112
P. procerum	00000010011000100100001001113210000002210112101
P. proliferum	01000110001000100200203122100100000000-10112101
P. rufisetum	0100000000?000100200211102200100001 00??10112101
P. rutteri	?10011100010011012000031 3-------00010221011212
P. semipellucidum	00000110001000100200101112100100000002210112101
P. sinense	00100000021001100100001102000100 0??000-10112101
P. spinulosum	13010-12001-----021-00-13-------011000-10112101
P. subtortile	0100001000?000100200101102100100000002210112112
P. subulatum	02000110011000100100001100000100000002210112101
P. tahitense	02000010001000100100001100200110000002210112101
P. tortile	01000000001000100100001100100120000002210112111
P. tubulosum	02000010001000100100001100200120000002210112101
P. urnigerum	00100010000100100000001000101101001000-10112101
P. usambaricum	01000110001000100100001102000100000002210112101
P. volvatum	01100010001000000100001000000100000000-10112101

Appendix 3. Command lines used for direct optimization with POY.

First analysis used the following commands:
poy -parallel -solospawn 15 mo.ss IT2a_1.ss IT2b t2 t4 t6 t8 -prealigned rp t1 t3 t7 t9
-noprealigned -molecularmatrix 111 -maxtrees 3 -holdmaxtrees 20 -random 10
-multibuild 15 -ratchettbr 3 -ratchettrees 2 -treefuse -fuselimit 25
-fusingrounds 1 -slop 1 -checkslop 5 -seed -1 -fitchtrees -noleading
-norandomizeoutgroup > ibp.out

mo.ss ITSa_1.ss IT2b t2 t4 t6 t8 rp t1 t3 t5 t7 t9 input filesnames, ibs.out output filename

In the second analysis, random value was 20, mutibuild 16, ratchettbr 5, and checkslop 8, otherwise as above.
In the third analysis, random and fuselimit was increased to 30, otherwise as above.
Fourth analysis was made with the same settings, but with 74 terminals

PHYLOGENETIC INFERENCES IN THE ORTHOTRICHOIDEAE (ORTHOTRICHACEAE, BRYOPHYTA) BASED ON VARIATION IN FOUR LOCI FROM ALL GENOMES[1]

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Abstract

Phylogenetic inferences based on analyses of the *trn*L-*trn*F region, *rps*4 gene (cpDNA), partial *nad*5 gene sequences (mtDNA), and partial sequences of the 26S (LSU, nrDNA) suggest that the Orthotrichoideae (i.e., the Zygodonteae and Orthotricheae) and the Zygodonteae are paraphyletic and that the genera *Zygodon* and *Orthotrichum* are not monophyletic. *Bryomaltaea obtusifolia* composes, with *Leratia neocaledonia* and *Orthotrichum exiguum,* the sister group to the Macromitrioideae. These species are now accommodated within a single genus, and *B. obtusifolia* and *O. exiguum* are transferred to *Leratia. Leptodontiopsis* is synonymized with *Zygodon,* and its four species (i.e., *L. elata, L. fragilifolia, L. macrocarpa,* and *L. orientalis*) are transferred to the latter. The status of *Pentastichella* and *Pleurorthotrichum,* which are both resolved with close affinities to *Zygodon* sensu stricto, must await further study. *Zygodon forsteri* is transferred to the genus *Codonoblepharon.* The circumscription of *Orthotrichum* is revised. The genus *Muelleriella* is merged with *Orthotrichum,* and its species transferred to it, requiring two new combinations (*Orthotrichum aucklandicum* [Vitt] Goffinet and *O. crassifolium* subsp. *acutum* [Vitt] Goffinet). Subgenus *Orthophyllum* is excluded from *Orthotrichum* and recognized as the genus *Nyholmiella. Stoneobryum* and *Sehnemobryum* are sister taxa and form a clade most likely derived from a common ancestor shared with other Orthotricheae. The genus *Ulota* is most closely related to species of *Orthotrichum* with superficial stomata (except *Nyholmiella*), but the systematic status of this combined lineage requires the evaluation of the affinities of other representatives. The phylogenetic hypothesis proposed here suggests that the haploid chromosome number ($n = 6$) for the lineage of cryptoporous *Orthotrichum* species is likely the result of an aneuploid reduction rather than an ancestral state. Whether sunken stomata arose once or twice remains unclear due to the position of *Sehnemobryum,* a phaneroporous taxon, sister to the cryptoporous *Stoneobryum,* in a rather basal position within the tribe.

Key words: 26S, Bryophyta, *Leratia, nad*5, Orthotrichaceae, *Orthotrichum,* phylogeny, *rps*4, *trn*L-*trn*F region, *Ulota, Zygodon.*

The roughly 13,000 species of mosses (Crosby et al., 1999) are currently distributed among 116 families and 859 genera (Buck & Goffinet, 2000). Some of the most speciose families comprise generic complexes wherein most species are accommodated in one genus from which a suite of smaller genera have been segregated based on the presence of one or more unique or unusual characters and hence are defined primarily on patristic distances from their putative relative. Whether such monospecific or otherwise species-poor genera compromise the monophyletic nature of the speciose genera has in many cases remained untested. Here we present phylogenetic inferences in the Orthotrichaceae aimed at elucidating the phylogenetic significance of species-poor but phenetically isolated genera within the Orthotrichoideae sensu Goffinet and Vitt (1998).

The family Orthotrichaceae comprises approximately 850 species (Crosby et al., 1999) distributed

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Table 1. Generic classification, generic diversity (in parentheses, following Crosby et al., 1999), and date of publication of genera of the Orthotrichaceae adapted from Goffinet and Vitt (1998).

Orthotrichoidea	
Zygodonteae (Schimper) Goffinet, in Goffinet & Vitt (1998)	
Bryomaltaea Goffinet (1), in Goffinet & Vitt (1998)	
Codonoblepharon Schwaegerichen (2 [8]), Spec. Musc. Suppl. 2(1): 142, 1824.	
Leptodontiopsis Brotherus (4), in Mildbr., Wiss. Ergebn. Deutsch. Zentr. Afr. Exp. 2: 146. 1910.	
Leratia Brotherus. (1), Öfvers. Finska Vetensk.-Soc. Føohr. 51A(17): 14, 1909.	
Pentastichella C. Müller (2), Öfvers. Finska Vetensk.-Akad. Handl. 47:1. 1905.	
Pleurorthotrichum Brotherus (1), Öfvers. Finska Vetensk.-Akad. Handl. 47:1. 1905.	
Zygodon W. J. Hooker & Taylor (91), Musc. Brit. 70. 1818	
Orthotricheae	
Muelleriella Dusén (4), Bot. Not. 1905: 304. 1905.	
Orthotrichum Hedwig (145), Spec. Musc.: 162. 1801.	
Sehnemobryum Lewinsky-Haapasaari & Hedenäs (1), The Bryologist 101: 549. 1998.	
Stoneobryum Norris & Robinson (2), The Bryologist 84: 96. 1981.	
Ulota Mohr (57), Ann. Bot. 2: 540. 1806	
Macromitrioideae Brotherus, Nat. Pfl. 11(2): 1925.	
Schlotheimieae Goffinet, in Goffinet & Vitt (1998)	
Schlotheimia Bridel (121), Sp. Musc. 2: 16. 1812.	
Macromitrieae	
Cardotiella Vitt (7), J. Hattori Bot. Lab. 49: 101. 1981.	
Ceuthotheca Lewinsky-Haapasaari (1), Lindbergia 19: 18. 1994.	
Desmotheca Lindberg (2), J. Lin. Soc. Bot. 13: 184. 1872.	
Florschuetziella Vitt (2), Bryologist 82: 16. 1979.	
Leiomitrium Mitten (1), Phil. Trans. Roy. Soc. Lond. 168: 390. 1879.	
Macrocoma (C. Müller) Grout (10), Bryologist 47: 4. 1944.	
Macromitrium Bridel (368), Mant. Musc. 132. 1819.	
Matteria Goffinet (2), in Goffinet & Vitt (1998)	
Synonymized taxa:	
Bryodixonia Sainsb. (= *Ulota*)	Goffinet & Vitt (1998)
Orthomitrium Lewinsky-Haapasaari & Crosby (= *Orthotrichum*)	Allen (2002)
Pleurozygodontopsis Dixon (= *Zygodon*)	Goffinet 1998
Excluded taxa:	
Amphidium Schimp. (Dicranaceae)	Goffinet et al. (1998)
Kleioweisiopsis Dixon (Ditrichaceae)	Goffinet (1998)
Octogonella Dixon (= *Rhachithecium* Broth. ex Le Jolis, Rhachitheciaceae)	Goffinet (1998)
Trigonodictyon Dixon & P. Varde (= *Grimmia* Hedw., Grimmiaceae)	Goffinet & Greven (2000)
Uleastrum Buck (Rhachitheciaceae)	Goffinet et al. (1998)

among 12 genera (Goffinet & Vitt, 1998). The Orthotrichaceae belong to the diplolepideous mosses, and are typically distinguished by their isodiametric, incrassate upper leaf cells, the lack of a central strand of conducting cells in the stem, a differentiation of such cells in the seta, a thicker outer peristomial layer, and the absence of ventral trabeculae on the exostome (Goffinet, 1997). Many species produce ribbed capsules, which bear a hairy calyptra prior to dehiscence. Perichaetia are borne at the apex of stems or erect branches. Brotherus (1925) and Vitt (1982) placed the acrocarpous species in the Zygodontoideae and the Orthotrichoideae, depending on the calyptrae being cucullate or mitrate, respectively. The Macromitrioideae comprised cladocarpous species with mitrate calyptrae. The position of the perichaetia is, however, not constant in either of these putative lineages: *Pentastichella* (Zygodontoideae) and *Ulota* (Orthotrichoideae) comprise cladocarpous taxa, and within the Macromitrioideae, at least *Matteria papillosa* is distinguished by producing female sex organs at the apex of stems or branches (Goffinet, 1997). Goffinet and Vitt (1998) proposed a revised classification of the Orthotrichaceae, based on inferences from *rbc*L sequence data (Goffinet et al., 1998) and from morphological data (Goffinet, 1997). They recognized two subfamilies, the Orthotrichoideae and the Macromitrioideae, and two tribes (Zygodonteae and Orthotricheae, Schlotheimieae and Macromitrieae, respectively) within each of these (Table 1).

The genera *Macromitrium* (with 368 accepted names fide Crosby et al., 1999), *Orthotrichum* (145 spp.), *Schlotheimia* (121 spp.), *Ulota* (57 spp.), and *Zygodon* (91 spp.) accommodate nearly 93% of the familial species diversity (Table 1). These genera were erected between 1801 and 1819, though only the circumscription of *Schlotheimia* has remained

stable. Indeed, 16 genera have subsequently been segregated from the other speciose genera. These segregates comprise few species, with *Muelleriella* and *Cardotiella* representing the most speciose of the new genera, having four (Vitt, 1976, 1979) and seven taxa (Vitt, 1979; Goffinet, 1996), respectively. Some taxonomic concepts have rapidly been abandoned (e.g., *Codonoblepharon* Schwaegr.), whereas others (e.g., *Muelleriella* Dúsen) have been adopted by some authors (Vitt, 1979) but rejected by others (Lewinsky, 1977).

Most recently, Goffinet (1997) tested the circumscription and the relationships of genera within the Orthotrichaceae against a criterion of monophyly, using phylogenetic inferences based on variation in morphological and molecular characters. Speciose genera were represented by two or more taxa. Most parsimonious reconstructions based on *rbc*L sequence data suggested that *Macromitrium*, *Orthotrichum*, *Ulota*, and *Zygodon* as defined by Vitt (1982) composed paraphyletic or polyphyletic lineages (Goffinet et al., 1998). These hypotheses were congruent with inferences based on gametophytic and sporophytic characters (Goffinet, 1997), leading Goffinet and Vitt (1998) to synonymize some names (e.g., *Bryodixonia* Sainsb. with *Ulota* Mohr), resurrect other segregate genera (e.g., *Codonoblepharon* Schwaegr. from *Zygodon* Hook. & Wilson), or erect new genera (e.g., *Bryomaltaea* for *Zygodon* sect. *Obtusifolii* Malta, and its sole species, *Z. obtusifolius*). The genus *Orthotrichum* was resolved as paraphyletic using either generational source of characters for reconstructing lineages. The r*bc*L sequences were only available for three species of *Orthotrichum* and three of *Ulota* (including *Bryodixonia*). Support for relationships was weak, except for the sister-group association between *O. lyellii* and *Ulota*. Representatives of all generic segregates were examined for their morphological characters (Goffinet, 1997). Under the most-parsimonious reconstructions, the genera *Muelleriella*, *Orthomitrium* Lewinsky-Haapasaari & Crosby (1996), *Stoneobryum* Norris & Robinson (1981), and *Ulota* s.l. occupied nested positions within *Orthotrichum*. To merge all these genera into a single genus would have resulted in a lineage whose morphological diversity far exceeded that of other genera within the family, by including taxa with smooth or papillose cells, with or without a differentiated basal border, superficial or sunken stomata, and large or small calyptrae. Consequently, systematic concepts within this complex were not revised. Similarly, the paraphyly of *Zygodon* s. str. (i.e., the lineage of papillose-leaved species of *Zygodon*) that was caused by the nested position of *Pleurorthotrichum*, *Pentastichella*, and *Leptodontopsis* was not further addressed. This cautionary approach was dictated by repeated accounts of phylogenetic reconstructions corresponding to gene genealogies rather than to species trees (see review in Doyle, 1992).

In this study, the hypotheses that *Orthotrichum* and *Zygodon* sensu Vitt (1982, 1984) and Lewinsky (1990, 1993) are paraphyletic assemblages are tested against phylogenetic inferences based on variation in sequences of four loci: the *trn*L-*trn*F region and the *rps*4 gene from the chloroplast genome, the *nad*5 gene intron from the mitochondrial genome, and partial sequences of the nuclear gene encoding the large subunit of the ribosomal RNA.

Material and Methods

Taxon and DNA sampling

Representatives of all genera recognized within the Orthotrichoideae by Goffinet and Vitt (1998) were included. In addition, two species of *Leptobryum* (Meesiaceae sensu Buck & Goffinet, 2000) were sampled to root the tree, and members of the Macromitrioideae were included to test the monophyly of the Orthotrichoideae (Table 2). Within the Orthotrichoideae, speciose genera (i.e., *Orthotrichum*, *Ulota*, and *Zygodon*) were represented by two or more species, belonging, in the case of the former genus, to different infrageneric taxa (Table 2).

DNA extraction follows the protocol described in Goffinet et al. (2004). Phylogenetic inferences were made based on variation in sequences of four loci, distributed across all three genomic compartments: the *trn*L-*trn*F region and *rps*4 gene (cpDNA), partial sequences of the *nad*5 gene (mtDNA), and the large subunit RNA gene (26S or LSU, nrDNA). Amplification and sequencing of the cpDNA loci follows Goffinet et al. (2004). The *nad*5 region was amplified and sequenced following Beckert et al. (1999) or, if this failed, we targeted a smaller fragment composed largely of the group I intron, using the primers 4F and 3R designed by Buck et al. (2004). The nuclear locus was amplified using the primers LS0F and LS8R using a nested approach relying on the preliminary amplicon obtained using 5.8SR and LS12R. The former two amplification primers also served for the sequencing reaction. Amplification was performed using HotMasterTaq polymerase (Eppendorf, Hamburg, Germany) (see Goffinet et al., 2004, for protocol).

Sequence editing and alignment

Products of the sequencing reactions were read using an ABI PRISM 3100 Genetic Analyzer, and

imported into Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, Michigan, U.S.A.). Sequences were edited for inaccurate base-calling prior to assemblage of forward and reverse strands into a consensus sequence. Edited sequences were entered into a matrix assembled in PAUP* version 4.0b10 (Swofford, 2002). Matrices were imported into SeAl v. 2.0 (Rambaut, 1996) for manual alignment. Sequences were deposited with GenBank (Table 2). The matrix was partitioned into four regions, each character set representing one locus.

PHYLOGENETIC ANALYSES

Inferences were made under the optimality criterion of either maximum parsimony (MP) or maximum likelihood (ML) using the software PAUP* 4.0b10 installed on G3 and G4 Apple Macintosh computers. Bayesian inferences were conducted using version 3.0 of MrBayes for Apple Macintosh (Huelsenbeck & Ronquist, 2002).

Analyses were performed on the complete data, each individual partition, or only the three cytoplasmic loci (*trn*L-*trn*F region, *rps*4 gene, and *nad*5 region). Regions of the *trn*L intron characterized by insertions and deletions were excluded from the analyses, but subsequently plotted on the tree. Under MP, all characters were given equal weight and all transformations considered equally parsimonious. For each data set, the following search strategy was implemented. First, 200 replicate searches, using nearest-neighbor-interchange (NNI) as the swapping method and implementing the steepest descent option and retaining 10 trees per replicate, was performed. Branches on all resulting trees were swapped using the tree bisection-reconnection (TBR) branch-swapping option, with a limit of 20,000 optimal trees to be retained, while the steepest descent option was turned off. If more than a single most parsimonious tree was saved, a 50% majority rule consensus tree was constructed. Support for branches was estimated using a non-parametric bootstrap approach (Felsenstein, 1985), whereby 300 pseudoreplicate matrices were constructed and each searched heuristically, twice with the steepest descent option invoked, for a total of 600 searches, resulting in a maximum of 30,000 trees saved (i.e., 50 per search). A 50% majority rule consensus tree was constructed from all trees saved. Nodes with bootstrap proportions (BP) exceeding 70% were considered well supported.

The model of molecular evolution that best fit the different data sets analyzed was estimated with a hierarchical likelihood ratio test using MrModeltest (Nylander, 2002), which is a simplified version of Modeltest 3.06 developed by Posada and Crandall (1998). Estimates of model parameters were fixed for subsequent searches under ML to the values proposed by MrModeltest. Heuristic searches using these parameter settings were repeated 10 times for each data set, using a TBR swapping algorithm. If multiple optimal trees were saved, a 50% majority rule consensus tree was constructed.

Two types of Bayesian phylogenetic analyses were performed on composite data sets whereby the complete data set was treated as a single partition or partitioned into individual loci (i.e., homogeneous vs. heterogeneous data sets). Individual partitions were also analyzed independently. Models for these partitions were implemented according to the results obtained using the likelihood ratio test (see above). Each analysis was based on 1,000,000 generations using four chains and flat priors. Model parameters were estimated and sampled every 50th generation. Trees saved prior to the likelihood score reaching stationarity were excluded from the set of trees used to construct consensus trees. The length of the burnin period was determined by visual inspection of the plot of the −ln likelihood score versus the number of generations. In all cases, this period was estimated to span for no more than 50,000 generations. Nodes with significant posterior probabilities (PP) were determined by constructing a 95% consensus tree of 19,000 trees. Under an assumption of heterogeneity of the data set, model parameters for each partition were allowed to vary independently. Trees and parameters were estimated every 50th generation.

Incongruence among data sets was assessed by comparing nodal support (either BP or PP) on optimal trees or their consensus tree. For example, if taxon A was resolved in clade A with a BP of 80% based on inferences on locus I, but reconstructed as a member of clade B with a BP of 75% based on locus II, the phylogenetic signal extracted from these loci was considered incongruent.

RESULTS

SIZE OF DATA SET

Sequences of all four loci were obtained for all taxa except for *Leratia neocaledonica* (*trn*L-*trn*F region, *rps*4, and LSU loci missing) and for *Groutiella chimborazensis*, *Orthotrichum exiguum*, *Pleurorthotrichum chilense*, *Zygodon forsteri*, and *Z. inermis*, for which the 26S LSU locus could not be amplified. Hence, the complete matrix was composed of 34 taxa and 130 sequences, of which 120 were newly contributed (Table 2). For *Muelleriella crassifolia*, only the first 700 of the 1200 base pairs

Table 2. GenBank accession numbers for sequences of the *trn*L-*trn*F region, the *rps*4 gene, the *nad*5 intron, and the partial 26S locus for the taxa of Orthotrichaceae and *Leptobryum* included in phylogenetic analyses. All specimens deposited at DUKE unless otherwise indicated.

Taxon	Voucher	GenBank accession No.			
		*trn*L-*trn*F	*rps*4	*nad*5	26S
Orthotrichoideae					
Orthotricheae					
Muelleriella crassifolia (Hook. f. & Wilson) Dusén	*Ochyra 367*	AY636018	AY618377		
	Goffinet 7005 (herb. Goffinet)			AY618400	
	Goffinet 6911 (herb. Goffinet)				AY621524
Orthotrichum					
subg. Gymonoporus (Braithw.) Limpr. sect. Affinia					
O. affine Schrad. ex. Brid.	*Vitt Exs. 41*[1]	AY636021	AY618365	AY618403	AY621527
subg. Gymnoporus sect. Leiocarpa Molendo					
O. lyellii Hook. & Taylor	*Schofield 95744*	AY636023	AY618367	AY618405	AY621529
subg. Pulchella (Schimp.) Vitt sect. Diaphana Ventori					
O. alpestre Hornsch. ex B. S. G.	*Vitt Exs. 47*	AY636017	AF306971	AY618399	AY621523
O. assimile Müll. Hal.	*Goffinet 5618*	AY636020	AY618364	AY618402	AY621526
O. macrocephalum F. Lara, Garilleti & Mazimpaka	*Albertos et al. s.n., 1996* (herb. Goffinet)	AY636015	AY618363	AY618397	AY621521
subg. Orthotrichum					
O. anomalum Hedw.	*Vitt Exs. 43*	AY636019	AF306973	AY618401	AY621525
subg. Exiguifolium Vitt					
O. exiguum Sull. in Gray	*Crosby 16042* (MO)	AY636034	AY626223	AY618416	
subg. Phaneroporum Delogne					
O. laevigatum J. E. Zetterst.	*Vitt Exs. 46*	AY636022	AY618366	AY618404	AY621528
subg. Orthophyllum Delogne					
O. obtusifolium Brid.	*Schofield 98451*	AY636014	AF306979	AY618396	AY621520
Orthomitrium tuberculatum Lewinsky-Haapasaari & Crosby	*Crosby 16040* (MO)	AY636016	AY618368	AY618398	AY621522
Sehnemobryum paraguense (Besch.) Lewinsky-Haapasaari & Hedenäs	*Vitt 21097* (ALTA)	AY636013	AY618382	AY618395	AY621519
Stoneobryum mirum (Lewinsky) Norris & H. Rob.	*Magill 7054* (MO)	AY636012	AY618381	AY618394	
Stoneobryum bunyaense Norris & H. Rob.	isotype (herb. Norris)				AY621518

Table 2. Continued.

Taxon	Voucher	GenBank accession No.			
		*trn*L-*trn*F	*rps*4	*nad*5	26S
Ulota crispa (Hedw.) Brid.	*Goffinet 4535*	AY636026	AF306972	AY618408	AY621532
U. hutchinsiae (Sm.) Hammar	*Goffinet 6512* (CONN)	AY636025	AY618370	AY618407	AY621531
U. phyllantha Brid.	*Schofield 100736*	AY636024	AY618369	AY618406	AY621530
Zygodonteae					
Bryomaltaea obtusifolia (Hook.) Goffinet	*Allen 12284*	AY636004	AY618358	AY618386	AY621513
Codonoblepharon pungens (Müll. Hal.) A. Jaeger	*LaFarge-England 8097* (herb. Goffinet)	AY636005	AY618383	AY618387	AY621514
Leptodontiopsis fragilifolia Broth.	*Pócs 9225/AZ* (herb. Goffinet)	AY636010	AY618374	AY618392	AY621517
Leratia neocaledonica Broth.	*Crosby 14185* (MO)			AY635998	
Pentastichella pentasticha (Mont.) Müll. Hal. ex Thér.	*Goffinet 5489*	AY636009	AY618373	AY618391	AY621516
Pleurorthotrichum chilense Broth.	*Goffinet 5320*	AY636011	AY618375	AY618393	
Zygodon bartramioides Malta	*Goffinet 5476*	AY636007	AY618371	AY618389	AY621515
Z. forsteri (Dicks. ex With.) Mitt.	*Long 9025*	AY636006	AY618376	AY618388	
Z. inermis Malta	*Goffinet 5410*	AY636008	AY618372	AY618390	
Macromitrioideae					
Schloteimieae					
Schlotheimia torquata (Sw. ex Hedw.) Brid.	*Allen 11979*	AY636027	AY618378	AY618409	AY621533
Macromitrieae					
Cardotiella quinquefaria (Hornsch.) Vitt		AY636032	AY618362	AY618414	AY621538
Desmotheca apiculata (Dozy & Molk.) Lindb.	*Arts 18/01* (herb. Goffinet)	AY636028	AY618379	AY618409	AY621534
Groutiella chimborazensis (Spruce ex Mitt.) Florsch.	*Streimann 21924*	AY636033	AY618380	AY618415	
Leiomitrium plicatum (P. Beauv.) Mitt.	*Goffinet 823* (herb. Goffinet)	AY636029	AY618359	AY618411	AY621535
Macrocoma tenuis (Hook. & Grev.) Vitt	*Arts 113/06* (herb. Goffinet)	AY636031	AY618361	AY618413	AY621537
Matteria gracillima (Thér.) Goffinet	*Arts 04/04* (herb. Goffinet) *Goffinet 7197a* (herb. Goffinet)	AY636030	AY618360	AY618412	AY621536
OUTGROUP					
Leptobryum stellatum (Herzog) Broth.	*Lewis 87-1222-d6*	AY501423	AY306991	AY618384	AY621511
L. wilsonii (Mitt.) Broth.	*Goffinet 5608*	AY501424	AY306992	AY618385	AY621512

[1] The number following "Vitt Exs." refers to the number of the species in the Orthotrichaceae Boreali-Americane Exsiccatae distributed by Vitt (1992).

Table 3. Length and proportion of variable sites in the *trn*L-*trn*F region, the *rps*4 gene, and the partial *nad*5 and 26S genes in the Orthotrichaceae (i.e., excluding the outgroup).

	Total length	Variable	Parsimony informative
*trn*L-*trn*F	345	120	82
*rps*4	571	147	78
*nad*5 (mainly intron)	1861	290	81
26S (partial)	825	115	61
cytoplasmic	2777	557	241
combined	3602	672	302

(bp) of the typical LSU amplicon could be sequenced. The *nad*5 sequences of *Matteria, Leptodontiopsis, Stoneobryum,* and *O. exiguum* cover mainly the intron only (± 1000 bp), and hence are about 700 bp shorter than those of the other taxa included.

For *Stoneobryum mirum* we were unable to amplify and sequence the 26S locus, but obtained the sequences from the only other species of the genus, *S. bunyaense.* In the case of *Muelleriella crassifolia,* loci from the three genomic compartments were obtained from three distinct specimens (Table 2).

Following the alignment of the sequences, the final matrix comprised 4114 characters. Regions flanking the primer annealing sites, as well as regions of ambiguous alignment across taxa, were excluded from all analyses. The size and levels of variation of the various data sets analyzed is shown in Table 3. Taxa with missing sequences were omitted from analyses of individual loci (e.g., *O. exiguum* is excluded from inferences based on LSU alone), but retained in analyses based on multiple loci.

Likelihood ratio tests led to the following models of sequence evolution to be implemented in the Bayesian and ML analyses, with the parameter values fixed for inferences under ML. For the complete data set (*trn*L-*trn*F, *rps*4, *nad*5, LSU) treated as a single character partition, the General-Time-Reversible model (Rodriguez et al., 1990) with a proportion of invariant sites and a discrete gamma distribution of rates (GTR + I + G) was selected with base frequencies set to 0.3010 (A), 0.1812 (C), and 0.2132 (G), character transformation rates set to 1.2460 (A–C), 3.4278 (A–G), 0.5339 (A–T), 0.9417 (C–G), 4.2831 (C–T), shape parameter set to 0.8443, and 48.40% of the sites considered to be invariable. For the combined set of cytoplasmic sequences the same model was implemented and the parameters fixed to the following values in the ML search: base frequencies set to 0.3215 (A), 0.1632 (C), and 0.1756 (G), character transformation rates set to 1.4004 (A–C), 4.2712 (A–G), 0.5022 (A–T), 0.7358 (C–G), 4.0805 (C–T), with shape parameter set to 0.9459, and 38.78% of the sites considered to be invariable. For the set of partial 26S sequences, the settings for GTR + G + I model were: base frequencies set to 0.2884 (A), 0.1877 (C), and 0.2197 (G), six categories of character transformation, with relative rates set to 1.485 (A–C), 3.6836 (A–G), 0.4592 (A–T), 1.0896 (C–G), 4.9083 (C–T), shape parameter of the gamma distribution of rates of evolution set to 0.8353, and the proportion of invariable sites to 51.9%. The proportion of invariable sites was considered insignificant for models selected for the remaining individual character partitions. For the ML search based on the *rps*4 sequences, the following settings were used for the GTR + G model: base frequencies set to 0.4174 (A), 0.1258 (C), and 0.1327 (G), six categories of character transformation, with relative rates set to 0.9519 (A–C), 2.9524 (A–G), 0.3097 (A–T), 0.715 (C–G), 3.011(C–T), and shape parameter of the gamma distribution of rates among sites set to 0.8875. For inferences based on the *nad*5 locus, parameter values for the GTR + G model were fixed as follows: base frequencies set to 0.2758 (A), 0.1792 (C), and 0.1962 (G), six categories of character transformation, with relative rates set to 1.6465 (A–C), 5.1458 (A–G), 0.4538 (A–T), 0.9547 (C–G), 5.5812 (C–T), and shape parameter of the gamma distribution of rates among sites set to 0.5926. The model invoked for inferences from the *trn*L-*trn*F sequences was the Hasegawa-Kishino-Yang model (Hasegawa et al., 1985) with a discrete gamma distribution of rates among sites (HKY + G) with the parameters set to: frequency of 0.38100 (A), 0.1259 (C), and 0.1586 (G), with shape parameter set to 0.4368, and the transition/transversion ratio set to 1.9123, and kappa set to 4.633075.

INFERENCES UNDER MAXIMUM PARSIMONY

Analysis of the combined data set yielded 6 optimal trees (length: 1158 steps; CI of informative characters [CI_{IC}]: 0.619; RI: 0.792). Excluding the 26S sequences resulted in 4 trees of 927 steps characterized by a CI_{IC} of 0.655 and an RI of 0.815. Inferences from (1) 26S alone resulted in 45 trees, each 207 steps long (CI_{IC}: 0.579; RI: 0.788); (2) *nad*5 alone yielded 20,000 trees of 403 steps (CI_{IC}: 0.700; RI: 0.838); (3) *trn*L-*trn*F alone led to 57 trees, each 234 steps long (CI_{IC}:0.691; RI: 0.813); and (4) *rps*4 alone resulted in 18 trees of 273 steps (CI_{IC}: 0.635; RI: 0.836).

INFERENCES UNDER MAXIMUM LIKELIHOOD

Each of the six analyses yielded the single optimal tree. The negative log likelihood (−ln) for these were: 12189.52 (combined data set); 9519.56 (plastid loci only); 2306.84 (*rps*4); 1648.27 *trn*L-*trn*F; 5085.65 (*nad*5); and 2413 (26S).

TOPOLOGICAL CONGRUENCE UNDER DIFFERENT OPTIMALITY CRITERIA

We compared the Bayesian posterior probabilities and MP bootstrap values of clades for conflicting high levels of support (i.e., BP greater than 70%, PP greater than 95%). For none of the data sets analyzed (i.e., the combined data, or any partition thereof) were conflicts in significant support for individual branches detected. Thus, although some nodes may be characterized by only one high support value (e.g., a high BP value but low PP score), the consensus topologies were not in conflict (i.e., level of support may be inconsistent but not incongruent at the levels of significance considered here). A similar parallelism has been shown previously (Cox et al., 2004), and thus our observations support the view that the two support parameters (BP and PP) may be complementary, in that they offer different insights into the nature of the characters (Alfaro et al., 2003).

CONGRUENCE AMONG DATA PARTITIONS

Assessment of topological incongruence among the four individual data sets based on comparison of significant support for branches revealed that the phylogenetic signal was congruent among the three cytoplasmic loci except for the affinities of *Codonoblepharon pungens*, which is resolved, based on *nad*5, within the Orthotricheae (BP = 79%, PP = 100%) rather than sister to *Z. forsteri* (all other inferences, Fig. 1), and incongruent between the cytoplasmic and nuclear DNA inference with regard to the placement of *Sehnemobryum* and *Stoneobryum* (basal in the Orthotrichaceae based on LSU data, and basal in the Orthotricheae based on the combined cytoplasmic data). The 70% majority-rule consensus tree of the bootstrap trees generated when analyzing the full data set is fully congruent with that obtained from analyzing the combined plastid-mitochondrial data. Optimal trees obtained under maximum likelihood analysis of individual character partitions deviated from that of the combined data sets, particularly in the case of inferences from LSU and *nad*5 sequences. The ML tree obtained based on *nad*5 is similar to the Bayesian and the MP consensus trees in that *Codonoblepharon* is resolved in a nested position within the Orthotricheae, but differs in the position of *O. obtusifolium*, which is included in the clade of phaneroporous *Orthotrichum* and *Ulota*. The latter taxa share virtually identical *trn*L-*trn*F sequences, and yet are resolved as a polyphyletic group in the *trn*L-*trn*F analysis. Furthermore, *Sehnemobryum* and *Stoneobryum* are also not sister-taxa as in other analyses (Fig. 1). However, these hypotheses receive no significant support in terms of either BP or PP. The LSU-based ML tree is substantially different. Here the Macromitrioideae compose a grade to the Orthotrichoideae, except for *Sehnemobryum* and *Stoneobryum*, which form a sister group to the rest of the family. Only those branches longer than 0.009 substitutions per site are also found in the tree obtained from the combined data set, but even these clades received no support from either BP or PP.

PHYLOGENETIC INFERENCES WITHIN THE ORTHOTRICHOIDEAE

All analyses resolve the genus *Orthotrichum* as polyphyletic. Polyphyly is consistently accounted for by (a) the strong affinities of *O. exiguum* with *Bryomaltaea obtusifolia* and *Leratia neocaledonica* (Table 4); (b) the nested position of *Muelleriella* and *Orthomitrium* in a clade of *Orthotrichum* species with immersed stomata (Table 4); and (c) representatives of *Ulota* composing a monophyletic group with those species of *Orthotrichum* that bear superficial stomata (Table 4). The genus *Zygodon* sensu Goffinet and Vitt (1998) is also consistently resolved as a paraphyletic group, due to the inclusion of *Leptodontiopsis*, *Pleurorthotrichum*, and *Pentastichella*. A sister-group relationship of the latter two to *Zygodon* plus *Leptodontiopsis* cannot be excluded, since support values for their inclusion in *Zygodon* are low. Furthermore, *Zygodon forsteri* is, under most reconstructions (i.e., all but *nad*5), sister to *Codonoblepharon*. *Leratia neocaledonica*, for which only the *nad*5 locus could be sampled, is most closely related to *Bryomaltaea obtusifolia* and *O. exiguum* (BP_{nad5} = 100%, PP_{nad5} = 100). Furthermore, all analyses resolve *Bryomaltaea* closely related to the Macromitrioideae rather than the Orthotrichoideae, making this subfamily either paraphyletic (all cytoplasmic loci) or polyphyletic (LSU). The Orthotricheae sensu Goffinet and Vitt (1998) compose a monophyletic assemblage under most reconstructions except when inferences were made from the LSU region alone, whereby *Stoneobryum* and *Sehnemobryum* compose a lineage sister to the rest of the family (BP = 81%, PP = 100),

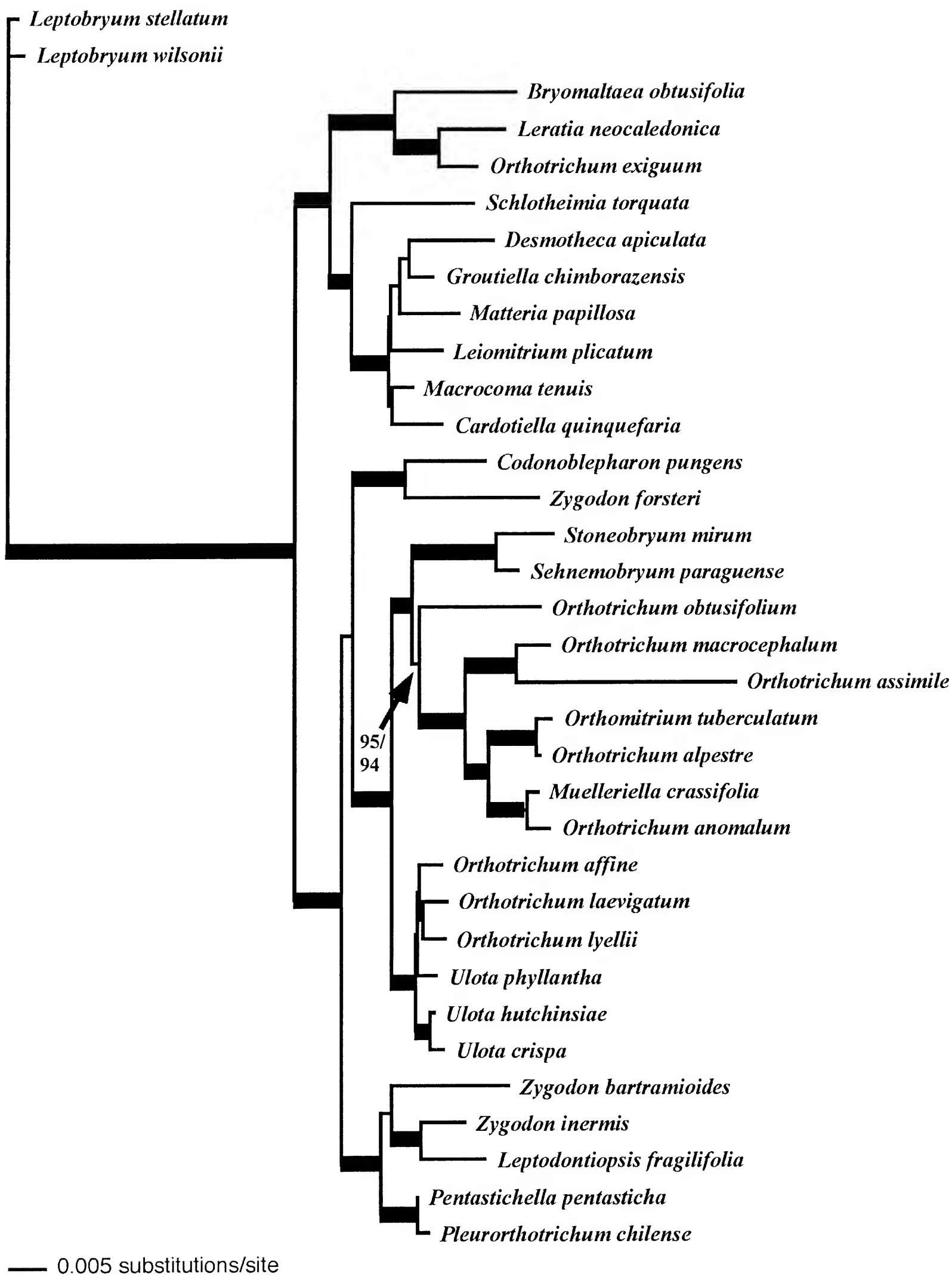

Figure 1. Phylogram of the single most likely tree ($-\ln L = 12{,}189.52303$) obtained under the assumption that the evolution of the combined sequences followed a single model of evolution, namely, a GTR + I + G model (see text for parameter values and tree statistics). Posterior probabilities were obtained under the same assumption (homogeneous) or that of data partitions (i.e., loci) evolving under different models (heterogeneous). Branches whose posterior probabilities are greater or equal to 95% are thickened. For one branch only one of the posterior probability satisfied that criterion, this branch is annotated with the homogeneous/heterogeneous posteriors. Note that *Stoneobryum mirum* is a composite entry; *trn*L-*trn*F, *rps*4, and *nad*5 are sequenced from this species, but the 26S locus was obtained only for *S. bunyaense*.

Table 4. Bootstrap (BP) and posterior probabilities (PP) characterizing the affinities of *Orthotrichum exiguum*, *Muelleriella crassifolia*, and *Orthomitrium tuberculatum*.

	Combined data						
	Homogeneous (BP/PP)	Heterogeneous (PP)	Cytoplasmic (BP/PP)	*nad*5 (BP/PP)	26S (BP/PP)	*trn*L (BP/PP)	*rps*4 (BP/PP)
O. exiguum + *Bryomaltaea obtusifolia* + *Leratia neocaledonica*[1]	100/100	100	100/	100/100	NA	79/100	/100
M. crassifolia + *Orthotrichum anomalum*	100/100	100	100/	79/< 95%[2]	82/< 95%[3]	100/100	98/100[3]
Orthomitrium tuberculatum + *O. alpestre*	100/100	100	100/	79/< 95%[2]	82/< 95%[3]	100/100	98/100[3]
Orthotrichum lyellii group + *Ulota* (3 species)	100/100	100	75/	< 70/< 95%[4]	86/100	< 70/ < 95%[4]	67 < 95%[4]

[1] *Leratia* is only included in analyses of the combined data set and the *nad*5 data.
[2] The *nad*5 data offer no support for relationships within the Orthotricheae.
[3] The *rps*4 and LSU data offer no support for relationships within an otherwise well-supported clade of *Orthotrichum* with immersed stomata.
[4] *nad*5, *trn*L, and *rps*4 yield no support for the affinities of *Orthotrichum* species with superficial stomata (excluding *O. obtusifolium*).

or when inferences were made based on *nad*5, whereby *Codonoblepharon* is resolved in a nested position within the Orthotricheae.

The phylogenetic relationships within the Orthotrichaceae based on inferences from the combined data set (Fig. 1) are congruent with the evolution of insertions or deletions in the *trn*L and the *nad*5 introns (Fig. 2). A seven to nine-bp polyT insertion (*trn*L) is shared by all members of the Macromitrioideae, *Bryomaltaea, Leratia,* and *Orthotrichum exiguum*. The Macromitrieae are defined by a 11-bp deletion (*trn*L), as well as by one- and seven-bp deletions and a three-bp insertion in the *nad*5. The Orthotrichoideae (excluding *Bryomaltaea, O. exiguum*, and *Leratia*) share a four-bp insertion in this intron. *Orthotrichum macrocephalum* and *O. assimile* share a seven-bp insertion (*trn*L). *Orthomitrium tuberculatum* and *O. alpestre* share a six- and a ten-bp insertion and a six-bp deletion (*trn*L). *Muelleriella* and *O. anomalum* both have an identical seven- and an eight-bp insertion (*trn*L). The clade comprising all taxa with immersed stomata and *S. paraguense* and *O. obtusifolium* is defined by a nine-bp deletion (*trn*L). The remaining species of *Orthotrichum* with superficial stomata share with *Ulota* a deletion of 23 bp in the trnL intron. *Leratia* and *O. exiguum* share a three-bp insertion in the *nad*5. In the Zygondonteae (excluding *Bryomaltaea* and *Leratia*) a ten-bp deletion (*trn*L) characterizes *Z. inermis, L. fragilifolia, P. pentastichum,* and *P. chilense*. These species and *Z. bartramioides* share a three-bp deletion in the *nad*5 intron.

Discussion

Congruence

Inferences made under the two optimality criteria (MP and ML) or for data partitions versus the combined data set were largely congruent, except for the position of *Codonoblepharon pungens* (incongruent for *nad*5) and *Sehnemobryum*/*Stoneobryum* (incongruent for LSU). Topological incongruence may be caused by lateral gene transfer through hybridization or by long branch attraction (Wendel & Doyle, 1998). It may also be an artifact of the data (e.g., Goffinet et al., 2003) or be due to inadequate assumptions in models of evolution. Hybridization may account for the incongruence between inferences based on nuclear sequences versus loci located in the cytoplasmic organelles. The ancestor to *Stoneobrym* and *Sehnemobryum* could have acquired its chloroplast and mitochondrial genome via hybridization with a member of the Orthotricheae. Alternatively, these taxa may have originated within the Orthotricheae and retained the nuclear genome from a

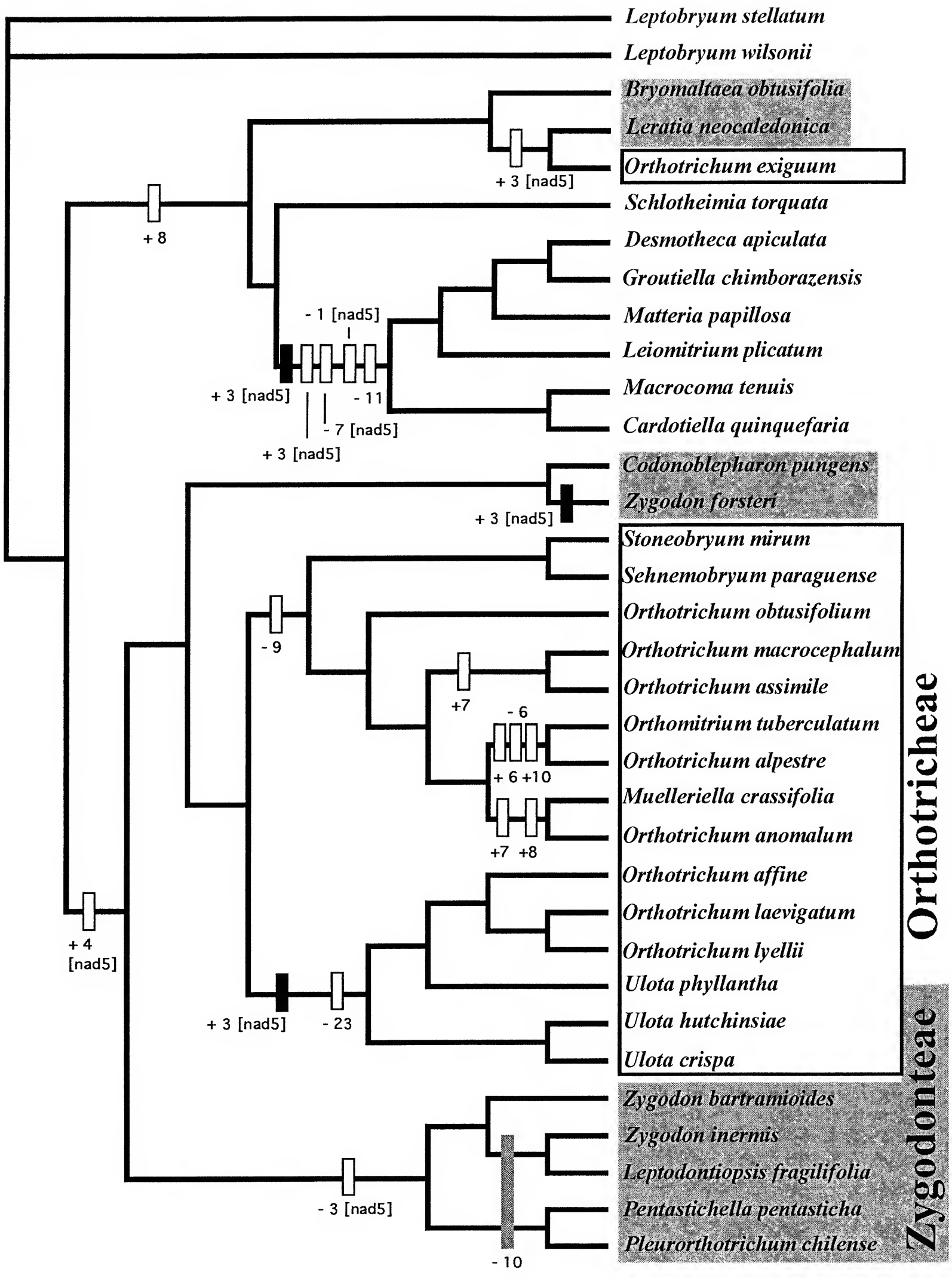

Figure 2. Distribution of unambiguous insertions and deletions in the *trn*L and *nad*5 introns based on the cladogram of the single most likely tree obtained under the assumption that the evolution of the combined sequences followed a single model of evolution, namely, a GTR + I + G model (see text for parameter values and tree statistics). Numbers refer to the gain or loss (−) in bp. Indels are located in the *trn*L intron unless otherwise marked. Colored boxes identify two potentially homoplasious indels (black and gray boxes, respectively). Note that *Stoneobryum mirum* is a composite entry; *trn*L-*trn*F, *rps*4, and *nad*5 are sequenced from this species, but the 26S locus was obtained only for *S. bunyaense*.

now extinct lineage of primitive Orthotrichaceae. Based on their mitrate calyptrae, orthotropic growth, sympodial branching, and acrocarpous perichaetial position, both *Stoneobryum* and *Sehnemobryum* are likely closely related to *Orthotrichum* (Norris & Robinson, 1981; Lewinsky-Haapasaari & Hedenäs, 1998), and hence belong without much doubt to the Orthotricheae, suggesting that this second hypothesis is more plausible, although the evidence is circumstantial at best. The possibility remains, however, that hybridization does not account for the incongruence, but that instead it may result from a long branch attraction with the outgroup due to passive weighting of linked characters. The large subunit of the ribosomal RNA exhibits a stringent secondary structure, composed of loops and stems (Capesius & Van der Peer, 1997). Mutations on one strand of the stem may lead to mutations on the complementary strand in order to maintain the secondary structure. Such linked changes may accentuate the observed degree of differentiation. It should be noted that conflict between inferences based on loci from the nuclear and cytoplasmic DNA has been reported before in bryophytes (e.g., Goffinet & Cox, 2000). Here, too, the nuclear sequence (18S) was not partitioned based on secondary structure. We are unable to assert whether inadequate data partitioning and character weighting is indeed a cause to the incongruence, but certainly it is a hypothesis that should not be dismissed. As for the case of *Codonoblepharon*, inference based on *nad*5 conflict with those made based on *trn*L-*trn*F, *rps*4, LSU (this study), and *rbc*L (Goffinet et al., 1998). *Codonoblepharon* exhibits all the morphological characters of the Zygodonteae and a nested position with the *Orthotrichum* complex appears irreconcilable with patterns in morphological variation, suggesting that the inference under *nad*5 may most likely be an artifact of the data. The combined data set offers strong support for the position of these taxa within the Orthotrichaceae, and it is this phylogenetic hypothesis that our subsequent discussion will focus on.

PHYLOGENETIC RELATIONSHIPS

The Zygodonteae sensu Goffinet and Vitt (1998) are consistently resolved as a paraphyletic group. Goffinet and Vitt (1998) had retained the genus *Bryomaltaea* close to *Zygodon*, despite earlier indications based on *rbc*L sequence data (Goffinet et al., 1998) that the genus may be closely related to the Macromitrioideae. Inferences based on other loci, including the mitochondrial and the nuclear genomes, are congruent with this hypothesis (Fig. 1). Analyses of the *nad*5 sequences also support the hypothesis proposed by Goffinet and Vitt (1998) that the New Caledonian genus *Leratia* is closely related to *Bryomaltaea*. Although we have been able to amplify and sequence only one of the four loci (*nad*5), inferences from this region are unambiguous (Table 4). Crum (1987: 603) considered *Leratia*, which was placed by Brotherus and Paris (in Brotherus, 1909) in the Macromitrioideae, "at the fringes of the subfamily, relatively close to the Orthotrichoideae" sensu stricto. That *Leratia*, as a representative of a clade also including *Bryomaltaea*, may mark the transition between the two subfamilies is here supported (cf. Figs. 1, 2).

The lineage composed of *Bryomaltaea obtusifolia* and *Leratia neocaledonica* is completed by *Orthotrichum exiguum*. Vitt (1971) had accommodated this species in its own subgenus, arguing that "phyletically, *O. exiguum* can be related to the remainder of the genus only remotely through a hypothetical ancestor" (Vitt, 1973: 106). The species differs from its congeners by a number of characters, and some of these characters are consistent with affinities to both *L. neocaledonica* and *B. obtusifolia*: the bulging laminal cells, with predominantly C-shaped papillae. The composite lineage of these three taxa differs from the Orthotrichoideae by their dextrorse seta, a character that is shared with the Macromitrioideae. Note though that the capsule in *O. exiguum* is immersed and the seta is very short with no indication of twisting. Goffinet and Vitt (1998) also mention prorate cells at the apex of the calyptrae in *B. obtusifolia* and *L. neocaledonica*; the calyptrae of *O. exiguum* is hairy, and not conspicuously papillose, except for the hairs and ridges. We are not aware of either prorate cells or papillose hairs occurring in the Macromitrioideae, and hence these features, along with the strictly acrocaprous development of perichaetia, distinguish the lineage composed of *Bryomaltaea*, *Leratia*, and *O.* subg. *Exiguifolium* Vitt. These three taxa are monospecific. We hereby propose to accommodate all three within a single genus, with the name *Leratia* Broth. (1909) taking priority. The required new combinations are made below (see Nomenclatural Implications, A). Whether *Leratia* should be accommodated in a separate subfamily should await critical examination of the phylogeny within the Macromitrioideae. Churchill and Linares (1996) elevated the Macromitrioideae to the family level, distinguishing it from the Orthotrichaceae by the cladocarpous development of female sex organs. In the light of the present phylogenetic inferences the Orthotrichaceae s. str. would be paraphyletic. Hence, we prefer to retain a broad concept of the family.

The remainder of the Zygodonteae forms either

a monophyletic or a paraphyletic group, with the combined data set strongly supporting the latter hypothesis. *Zygodon forsteri* is sister, under most assumptions, to the other smooth-celled species sampled, *Codonoblepharon pungens*. Such affinities had earlier been proposed by Malta (1926), who placed *Z. forsteri* within subgenus *Bryoides*, a taxon defined by smooth laminal cells. A monophyly of this lineage is only incongruent with inferences from the *nad*5 locus, whereby *C. pungens* appears nested within the Orthotricheae, a position very much inconsistent with patterns in morphological characters (Goffinet, 1997) and inferences from cpDNA (this study and Goffinet et al., 1998), and hence considered an artifact until confirmed by further studies. Goffinet and Vitt (1998) resurrected the genus *Codonoblepharon* to accommodate those species of *Zygodon* that lacked papillae on their laminal cells. *Zygodon forsteri*, which also has smooth cells, was retained within *Zygodon*. The species is restricted to western Europe, whereas all other species of *Zygodon* lacking papillae are endemic to the Southern Hemisphere. Goffinet and Vitt (1998) viewed *Z. forsteri* as having evolved from a papillose ancestor within *Zygodon* s. str. This hypothesis is here refuted on the basis of inferences from cpDNA, as well as the combined data set. Hence, the species is better accommodated under *Codonoblepharon* (see Nomenclatural Implications, B, below).

The affinities of *Codonoblepharon* remain ambiguous. Most optimal topologies resolved the revised Zygodonteae (i.e., *Codonoblepharon* and *Zygodon* s. str., and thus excluding the *Leratia* complex) as a paraphyletic group leading to the Orthotricheae. Given the sporophytic similarity between these two genera, we prefer to retain the Zygodonteae until further studies resolve the ambiguity. The Zygodonteae now accommodate only *Codonoblepharon* and *Zygodon*.

The paraphyly of *Zygodon* sensu Goffinet and Vitt (1998) results further from the nested position of *Leptodontiopsis*, and the potential inclusion of *Pleurorthotrichum* and *Pentastichella*. The genus *Leptodontiopsis* is disjunct between East Africa and Southeast Asia and is composed of four species that differ primarily in leaf characteristics (Dixon, 1918, 1935, 1938). *Leptodontiopsis fragilifolia*, the nomenclatural type species of the genus, is consistently resolved in a nested position within *Zygodon* sensu stricto. *Leptodontiopsis* shares with members of *Zygodon* the presence of small conical papillae on the surface of the upper laminal cells and a smooth cucullate calyptrae. Goffinet (1997) had proposed that this genus may be more closely related to *Pentastichella* and *Pleurorthotrichum*, with which it shares dimorphic basal cells (i.e., some basal cells are thick walled and porose). This feature may be more common in *Zygodon* (Malta, 1926), and hence should not serve a diagnostic character for a clade comprising these three genera. Potier de la Varde (1942) and Goffinet and Vitt (1998) diagnosed *Leptodontiopsis* by its smooth rather than ribbed capsules. Although most species of *Zygodon* seem to have capsules whose exothecial cells are differentiated into longitudinal bands of thick-walled cells, several species seem to have smooth urns (Malta, 1926). The long seta was also seen as a diagnostic character for *Leptodontiopsis* by Potier de la Varde (1942), but this feature may be correlated to the greater stature of the plants and is actually not restricted to this genus within the Zygodonteae (Malta, 1926). Hence, no character seems to consistently segregate the two genera, and therefore we support the proposal by Zander and Vitt (1979) to place the genus *Leptodontiopsis* in synonymy with the earlier name *Zygodon* (see Nomenclatural Implications, C, below).

Pleurorthotrichum and *Pentastichella* are also closely related to *Zygodon* sensu stricto. *Pentastichella* is distinguished from *Zygodon* on the basis of the five-ranked leaves and its creeping stems. Malta (1926) treated the genus as a section of *Zygodon* (as *Z.* sect. *Stenomitrium* Mitt.). Although he did not dispute the morphological differences, he argued against a generic recognition as proposed by Müller (1897, as *Pentastichella*) and Brotherus (1902, as *Stenomitrium*) because (1) *Zygodon bartramioides* composed an intermediate, linking this section to species of section *Euzygodon* C. Müller, and (2) because he had treated other morphologically distinct lineages (e.g., smooth-celled taxa) at the section level. Phylogenetic inferences based on morphology resolved *Pentastichella* in a nested position within *Zygodon* (Goffinet, 1997). Goffinet and Vitt (1998), who elevated the sections recognized by Malta as genera and followed Brotherus, accommodating *Z. pentastichum* in its own genus, *Stenomitrium*. As pointed out by Crosby (pers. comm.), the name *Pentastichella* is not an illegitimate name and hence has priority. Goffinet (1997) indicated that *Pentastichella* differs from species of *Zygodon* not only by its mode of branching (monopodial), and consequently its growth form (creeping) and the position of the perichaetia (cladocarpous), but also by the direction of the twisting of the seta, which is sinistrorse in other species of *Zygodon* s. str. versus dextrorse in *Pentastichella*. The latter was also considered allied to *Leptodontiopsis* and *Pleurorthotrichum* and distinct from *Zygodon* on the ba-

sis of the dimorphic basal cells, a feature, which is, however, also present among species of the latter genus (Malta, 1926). Our taxon sampling within *Zygodon* was limited, and the position of *Pentastichella* remains ambiguous, except for its sister-group relationship with *Pleurorthotrichum. Zygodon bartramioides* shares with *Pentastichella* five-ranked leaves as well as a well-developed exostome, which led Malta (1926) and Brotherus (1925) to consider this species a member of section *Stenomitrium* in *Zygodon.* The species differ, however, in the architecture of the endostome (reduced in Z. *bartramioides* vs. well developed in *P. pentasticha*) and the size of the spores (10.5 μm diam. vs. 26 μm, respectively, fide Malta, 1926). Griffin (1990) described *Z. bartramioides* as having creeping stems with erect branches terminated by perichaetia. We have not seen a differentiation of stems and branches as characteristic of *Pentastichella.* As mentioned above, *P. pentasticha* is the sole member of the Zygodonteae to be cladocarpous. This mode of perichaetial development is scattered across the family, being virtually constant among the Macromitrioideae, but also present in *Ulota* (Malta, 1927) and *Orthotrichum* (Norris et al., 2004). Thus, the sole character separating *Pentastichella* from species of *Zygodon* may be the twisting of the seta. Our voucher specimen for *Z. bartramioides* is fruiting and the distal portion of the seta is twisted to the left, and hence is dextrorse as in *Pentastichella.* It should be noted that the dextrorse seta also characterizes *Leratia,* which forms a sister group to the Macromitrioideae s. str. It is possible that this condition is plesiomorphic in the family, and has been transformed independently in the two subfamilial lineages of the Orthotrichaceae. Until the affinities of *Pentastichella* are further resolved we prefer to retain it as a distinct genus within the Zygodonteae.

Pleurorthotrichum has remained monospecific since its description (Brotherus, 1902). The affinities of this taxon have recently been addressed by Lewinsky-Haapasaari (1994), who considered *Pleurorthotrichum* related either to the Macromitrioideae (due to a putative placement of perichaetia on branches) or to the genus *Orthotrichum* (general habit and the presence, as in *O. lyellii* of a layer of differentiated cells around the central strand). Lewinsky-Haapasaari (1994) even suggested accommodating *Pleurorthotrichum* in its own subfamily, in part due to the endostomial architecture (Goffinet, 1996). By contrast, Goffinet (1997) rejected an affinity to the Macromitrioideae due to the acrocarpous rather than cladocarpous nature of the perichaetia and suggested, instead, that *Pleurorthotrichum* belonged to the Zygodonteae due to the presence of several small and even inconspicuous papillae on the laminal cells. This hypothesis is well supported by analyses of sequence data of the plastid genomes (LSU could not be obtained for this species). The sole species *Pleurothotrichum chilense* is sympatric with another southern American endemic, *Pentastichella pentasticha,* with whom it seems to share a common recent ancestor. The two species share five-ranked leaves and a dextrorse seta. None of these characters are, however, unique within the Zygodonteae, and thus diagnostic with regard to *Zygodon* s. str. (see above). *Pleurorthotrichum* is easily distinguished by long vegetative leaves, its differentiated perichaetial leaves, the hairy calyptrae, and the anatomy of the seta. A cucullate and hairy calyptra as well as differentiated perichaetial leaves are also known within *Zygodon* from the African *Z. trichomitrius* Hook. & Wilson (Malta, 1926). It is possible that *Pleurorthotrichum* should be regarded as a member of the genus *Zygodon,* or alternatively that the circumscription of a *Pentastichella–Pleurorthotrichum* lineage should be broadened to include pentastichous taxa of *Zygodon.* As advocated for *Pentastichella,* further critical testing of the monophyly of *Zygodon* is needed before systematic changes are proposed.

The Orthotricheae are currently defined by their mitrate calyptrae, whose outer cells are thick walled with round lumens and unistratose (Goffinet & Vitt, 1998; note that *O. exiguum,* here excluded from the genus [Nomenclatural Implications, A], was not seen by these authors). The circumscription of this lineage is supported by our analyses. By contrast, the monophyly of the speciose genus *Orthotrichum* is consistently rejected by all inferences. Although our sampling within *Orthotrichum* is limited, it reveals that phaneroporous species of subgenus *Phaneroporum* (e.g., *O. laevigatum*) and *Gymnoporous* (e.g., *O. lyellii*) share a common ancestor with *Ulota* (also phaneroporous) rather than with their cryptoporous congeners. Such a phylogenetic hypothesis had been proposed earlier by Goffinet et al. (1998) based on analyses of *rbc*L data, and is here confirmed based on additional loci. Two other phaneroporous species sampled, *O. obtusifolium* and *Sehnemobryum paraguense,* consistently exhibit closer ties to the cryptoporous taxa. This composite lineage further includes *Stoneobryum, Muelleriella,* and *Orthomitrium* (all cryptoporous). The bispecific *Orthomitrium* is endemic to China (Lewinsky-Haapasaari & Crosby, 1996; Tan & Jia, 1997) and was segregated based on a combination of unusual features and what was then considered a unique trait, namely, the puckered

mouth of the capsule. Allen (2002) already pointed out that this character is scattered within the family and should not serve to diagnose a distinct genus. Our inferences support his decision to transfer the two species of *Orthomitrium* to *Orthotrichum*, as *Orthotrichum jettae* Allen nom. nov. for *Orthomitrium tuberculatum* and *Orthotrichum schofieldii* (Tan & Jia Yu) Allen.

The genus *Muelleriella* comprises four species, all restricted to the subantarctic region. A generic recognition of *Muelleriella* was argued for by Vitt (1976, 1979) based on a unique combination of characters, including spores that are multicellular at the time of dehiscence and multistratose leaves with nearly smooth cells. Lewinsky (1977) initially rejected a generic segregation, considering that stomata had evolved only once in the family, and hence that species of *Muelleriella* were closely related to other cryptoporous species of *Orthotrichum*. Subsequently, Lewinsky (1993) adopted Vitt's concept, although she remained skeptical, particularly following her phylogenetic study (Lewinsky-Haapasaari & Hedenäs, 1998). Inferences from sequence data leave no doubt: *Muelleriella* is nested within a group of cryptoporous *Orthotrichum*. Although we have only been able to sample one species, it is likely considering the number of features the four species share (Vitt, 1976) that our observation can be extrapolated to the genus. To consider *Muelleriella* synonymous with *Orthotrichum* requires two new combinations (see Nomenclatural Implications, D, below).

Cryptoporous stomata also characterize the two species of *Stoneobryum*, a genus segregated from *Orthotrichum* by the following combination: immersed capsule, small hairy calyptrae covering only the operculum, and differentiated perichaetial leaves (Norris & Robinson, 1981). Although Lewinsky-Haapasaari and Hedenäs (1998) did include the species in their analyses that were focused on the infrageneric phylogeny of *Orthotrichum*, they did specify *Stoneobryum* as a member of the outgroup. Nevertheless, when multiple outgroups were specified, *Stoneobryum* exhibited strong affinities to members of the ingroup, in particular to *Sehnemobryum*, a monospecific genus with superficial stomata. When *Stoneobryum* was used as the sole outgroup, *Sehnemobryum* was resolved in a most basal position within *Orthotrichum*. A strong affinity between these two genera is not surprising in the light of their overall resemblance (Lewinsky, 1978; Lewinsky-Haapasaari & Hedenäs, 1998). Both genera are retained as distinct phylogenetic lineages.

Orthotrichum obtusifolium and *O. gymnostomum* (the latter was not included in this study) compose another subgenus of *Orthotrichum* (i.e., subg. *Orthophyllum*) that has also been treated as a distinct genus (i.e., *Stroemia* Hag., nom. illeg. replaced by *Nyholmiella* Holmen & Warncke in Damsholt et al., 1969). The species are best distinguished by their dioicous condition, leaves with erect to involute margins, and obtuse apices. None of these features uniquely define the subgenus though. Unlike most other taxa with superficial stomata, the basal cells of the leaves are thin walled and not nodose, and their haploid chromosome number is $n = 11$, rather than $n = 6$ (Vitt, 1973: 113; Lewinsky, 1993). However, "there are no character states which these two species have in common which are not found also in other species of the genus" (Vitt, 1973: 113). Subgenus *Orthophyllum* was regarded by Lewinsky (1993) as a sister group to the remainder of *Orthotrichum*, and Vitt (1971), too, considered it to be isolated from most of the other subgenera, although descending from a common ancestor (Vitt 1973). Phylogenetic inferences based on morphological characters yielded no unambiguous result, except that the subgenus consistently appeared nested within a clade comprising species of subgenus *Gymnoporus* (e.g., *O. affine*), *Phaneroporum* (e.g., *O. laevigatum*), and *Pulchella* (e.g., *O. alpestre*), and hence offer no support for recognizing subgenus *Orthophyllum* (Lewinsky-Haapasaari & Hedenäs, 1998). Similarly, inferences from *rbc*L sequence data offered little insight into the affinities of *O. obtusifolium* (Goffinet et al., 1998). By contrast, analyses of other loci provide strong support for *O. obtusifolium* to belong to a lineage comprising all cryptoporous Orthotrichaeae as well as *Sehnemobryum*. Our results are thus congruent with Vitt's (1971) view that the subgenus occupies a rather isolated position within the Orthotricheae. Since we retain *Sehnemobryum* and *Stoneobryum* as distinct genera, and considering their nesting position (based on the combined data set) between subgenus *Orthophyllum* and cryptoporous species of *Orthotrichum*, the two species of subgenus *Orthophyllum* are well accommodated in their own genus (i.e., *Nyholmiella* Holmen & Warncke in Damsholt et al.), and we hence follow Holmen and Warncke (in Damsholt et al., 1969). No nomenclatural changes are needed.

As indicated above, phaneroporous species of *Orthotrichum* belonging to subgenus *Phaneroporum* and *Gymnoporous* compose a sister group to or are nested within the genus *Ulota*, whose stomata are strictly phaneroporous. The monophyly of this group of *Orthotrichum* species is not well supported (Fig. 1). Similarly the monophyly of *Ulota* is ambiguous. *Ulota phyllantha* is consistently resolved

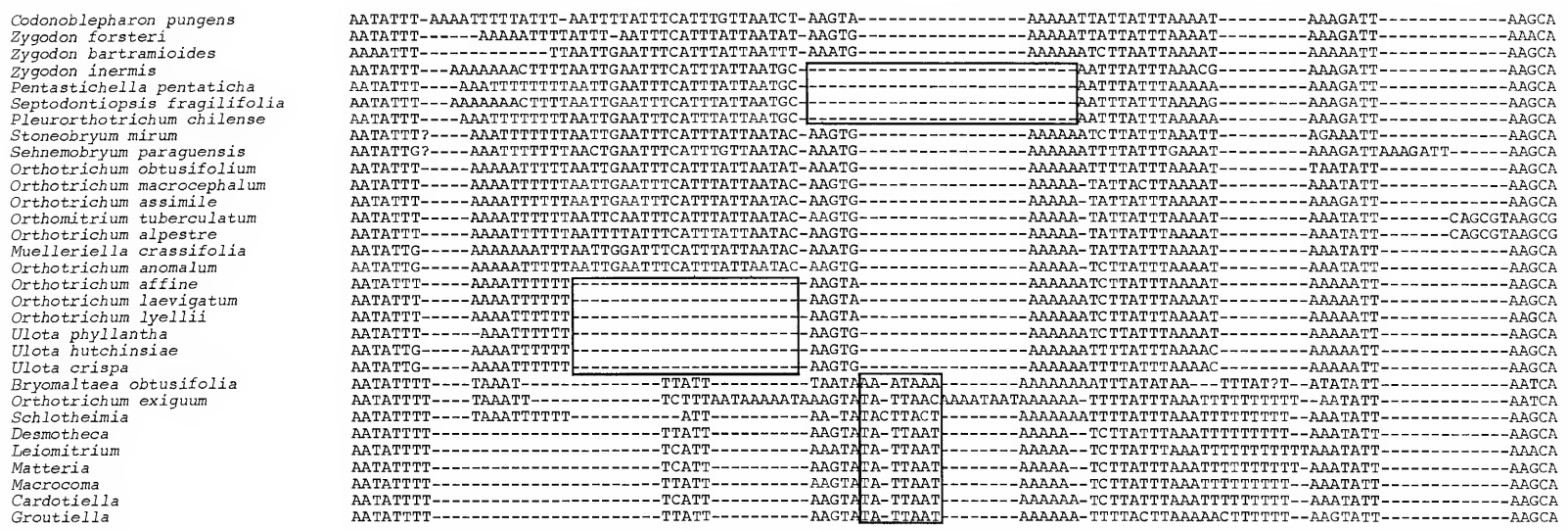

Figure 3. Aligned portion of the *trn*L intron. The whole segment was excluded from the analyses. The structure in the variation of the sequences matches, at least in part, the phylogenetic pattern retrieved based on the conserved portions of the sequences. Only the segments that are boxed have been plotted on the tree in Figure 2.

among optimal trees, as a sister taxon to the phaneroporous species of *Orthotrichum*, but the support for this hypothesis is virtually absent. It's worth noting that the taxa composing this clade are poorly differentiated in terms of their nucleotide sequences. The paucity of characters may explain the lack of resolution and support for the monophyly of either *Ulota* or this lineage of phaneroporous *Orthotrichum* species. The inferences presented here are congruent with those made by Goffinet al. (1998) using *rbc*L, whereby *Orthotrichum* is paraphyletic with *O. lyellii* sharing a common ancestor with *Ulota*. Lewinsky-Haapasaari and Hedenäs (1998) did include species of *Ulota* in their analyses, but designated these as outgroups. However, if the tree shown in their figure 5 (1998: 533) is rooted with *Stoneobryum*, *Ulota* would appear nested in *Orthotrichum* and more or less mark the split between the *Leiocarpa* group (phaneroporous species) and *Pulchella* group (cryptoporous species), and hence approximate the topology presented here (Fig. 1).

The shared ancestry of the phaneroporous species of *Orthotrichum* and *Ulota* is well supported by the nucleotide data. These taxa differ from other Orthotrichoideae by the loss of 23 nucleotides in the *trn*L intron (Fig. 3). Such molecular diagnosis is not matched by a morphological synapomorphy. The monophyly of this group of *Orthotrichum* species and *Ulota* finds no support based on sequence data. *Ulota* is characterized by the differentiation of basal marginal cells that are quadrate and have incrassate anticlinal walls, by flexuose to crisped leaves, and a calyptrae covering only half the capsule (e.g., Vitt et al., 1993). The distinction of these taxa becomes nebulous when certain species such as the South American *U. fulvella* Mitt. (not included here), which lack a conspicuously differentiated basal border, are considered (Malta, 1927). Whether this results from a reduction or is instead a real symplesiotypic state remains to be determined. Similarly some species of *Orthotrichum*, such as *O. pulchellum* Brunt., have flexuose rather than erect leaves, and hence also contribute to obfuscate the boundaries between the lineages. *Ulota* and the phaneroporous species of *Orthotrichum* may, however, differ consistently in one character, namely, their haploid number of chromosomes. These *Orthotrichum* species with superficial stomata share a chromosome number of $n = 6$ (Lewinsky, 1993), whereas species of *Ulota* have typically 10 or more chromosomes (see discussion below). Hence, we prefer to retain *Ulota* as distinct from these phaneroporous taxa. The genus *Orthotrichum* is a polyphyletic entity, despite the systematic changes proposed above, due to the position of the phaneroporous species allied to *Ulota*. Before a new genus is erected to accommodate the former, phylogenetic inferences should be based on a critical sampling of taxa representative of the morphological variation within *Orthotrichum* subg. *Gymnoporous* and *Ulota*.

CHARACTER EVOLUTION

The phylogenetic hypothesis proposed here based on inferences from a multigenomic set of data allows us to tentatively address the evolution of two characters considered significant in the diversification of the Orthotrichoideae, namely, the haploid number of chromosomes and the architecture of the stoma. Species of *Orthotrichum* with superficial stomata have a chromosome number of $n = 6$, though not all species have been characterized yet. By contrast, their congeners with immersed stomata have a number $n = 11$ (Vitt, 1971; Lewinsky, 1993). Vitt (1971) suggested that the latter arose via autoploidy

followed by an aneuploid reduction by one chromosome. Cytological data are not available for the majority of taxa (Fritsch, 1991); however, the correlation between stomatal architecture and *n* values in the genus *Orthotrichum* seems to be strong, except for subgenus *Orthophyllum* (i.e., *Nyholmiella*), whose taxa have superficial stomata but their cells hold 11 and not 6 chromosomes. This clade of two species is here consistently resolved as a sister group to the clade composed of taxa with immersed stomata and $n = 11$. Cytological data are even scarcer for other genera of the subfamily, especially exotic taxa (e.g., *Stoneobryum* and *Sehnemobryum*), where the numbers range between $n = 11$ and $n = 12$ (2 species counted, Fritsch, 1991) in *Zygodon* (s. str.) and between $n = 10$ and $n = 22$ (8 species counted, Fritsch, 1991) in *Ulota* (although one population of *U. japonica* was characterized by $n = 8$ by Yano (1951), a value inconsistent with a later count for another population, which had $n = 10$ (Yano, 1957)). Placed within our phylogenetic hypothesis, wherein species of *Orthotrichum* with $n = 6$ compose a crown group within a lineage whose species have 10 or more chromosomes, these observations suggest that taxa with $n = 6$ may be extreme aneuploids of an ancestral $n = 12$. Thus, $n = 6$ should not be considered plesiotypic within the Orthotrichoideae, the Orthotricheae, or the *Orthotrichum* complex as suggested by Vitt (1971) and Lewinsky (1993). Similar aneuploid reductions have been proposed to account for numbers such as $n = 7$ or 8 in the *Macromitrium* complex (Ramsay & Vitt, 1986). These authors also proposed that those species with $n = 12$ arose via polyploidization of an ancestor with $n = 6$. The cytological evolution in the Orthotrichaceae would thus involve chromosome doubling followed by a reduction. The question remains when the gametophytic diploidization took place: prior to the early diversification of the family, or independently in the Macromitrioideae and the Orthotrichoideae.

The second character of interest is the architecture of the stomata. The phylogenetic affinities of *Muelleriella* and *Orthomitrium* clearly refute the hypothesis that these taxa acquired immersed stomata independently, thereby supporting Lewinsky's (1977) hypothesis that this architectural type arose only once in the family. Immersed stomata do, however, also characterize *Stoneobryum*, a genus disjunct between South Africa and Australia. Although *Stoneobryum* may be part of the sister clade to the lineage of cryptoporic *Orthotrichum* species, it is most closely related to *Sehnemobryum*, a genus whose sole species has phaneroporic stomata. Given this phylogenetic hypothesis, immersed stomata could have arisen independently in *Stoneobryum* and the ancestor to the cryptoporic *Orthotrichum*, or have arisen once in their common ancestor and been lost in *Sehnemobryum*. We will formally address this hypothesis once the affinities of the remaining supraspecific taxa (e.g., *Callistoma* (Iwats. & Sharp) Lewinsky (cryptoporic)) have been determined.

Nomenclatural Implications

A. *Leratia*

We propose to accommodate *Bryomaltaea obtusifolia*, *Leratia neocaledonica*, and *Orthotrichum exiguum* within a single genus with the name *Leratia* Broth. (1909) taking priority. The transfer into the genus *Leratia* requires the two new combinations and leads to the following synonymies.

Leratia Broth., Öfv. Finska Vet. Soc. Förh. 51A: 14. 1909. Type: *Leratia neocaledonica* Broth.

Zygodon sect. *Obtusifolii* Malta, Latv. Univ. Raksti 6: 282. 1923. Syn. nov. Type: *Zygodon obtusifolius* Hook.

Orthotrichum subg. *Exiguifolium* Vitt, Nova Hedwigia 21: 705. 1973. Syn. nov. Type: *Orthotrichum exiguum* Sull.

Leratia obtusifolia (Hook.) Goffinet, comb. nov. *Zygodon obtusifolius* Hook., Musci Exo.: 159. 1820. *Bryomaltaea obtusifolia* (Hook.) Goffinet, syn. nov., Bryology for the Twenty-first Century: 151. 1998. TYPE: Nepal. *D. Gardner s.n.* (lectotype, BM designated by Lewinsky, 1990: 121, not seen).

Leratia exigua (Sull.) Goffinet, comb. nov. *Orthotrichum exiguum* Sull. in Gray, Man. Bot. No. U. States ed. 2: 633. 1858. TYPE: South Carolina. Santee Canal, Feb.–Apr. 1847, *Ravenel s.n.*, (holotype, FH-Sull, not seen).

B. *Codonoblepharon*

Zygodon forsteri is transferred to *Codonoblepharon* based on inferences from the combined data set.

Codonoblepharon forsteri (Dicks. ex With.) Goffinet, comb. nov. *Bryum forsteri* Dicks. ex With., Syst. Arr. Brit. Pla (ed. 4) 3: 798. 1801. *Zygodon forsteri* (Dicks. ex With.) Mitt., Ann. Mag. Nat. Hist ser. 2, 8: 321. 1851. TYPE: England. Walthamstow, on dead trunks of trees, *Forster s.n.* (holotype, presumably BM, not seen).

C. *LEPTODONTIOPSIS*

This genus is here synonymized with *Zygodon*, and hence its species are transferred to the latter. The type of the genus *Leptodontiopsis* is *L. fragilifolia* (Brotherus, 1910). This epithet is already used in the genus *Zygodon* (*Z. fragilifolius* Brotherus in Malta, 1926). Both species appear to have been described by Brotherus based on different specimens. Potier de la Varde (1942) first noticed the similarity between the species and treated the material described by Brotherus in Malta (1926) as a variety of the *L. fragilifolia* (*L. fragilifolia* var. *brotheri*), considering it to represent simply a phenotypic variant, characterized among others by longer papillae on the marginal cells. We have not seen the type specimens and hence will follow his concept and tentatively treat the two taxa as varieties. Although *L. fragilifolia* was described prior to *Z. fragilifolius*, the epithet *fragilifolia* is preoccupied. The variety "*brotheri*," used for *Z. fragilifolia* Brotherus (in Malta, 1926) by Potier de la Varde, should now be treated as variety *fragilifolia* (= *L. fragilifolia* var. *brotheri* P. de la Varde, Bulletin du Muséum 2e s. 14: 368. 1942), which is the type variety, and a new variety name must be proposed for what Brotherus (1910) treated as *Leptodontiopsis fragilifolia* and Potier de la Varde (1942) as *L. fragilifolia* var. *fragilifolia*.

Zygodon fragilifolius var. **papillosus** Goffinet nom. nov. based on *Leptodontiopsis fragilifolia* Brotherus, Wiss. Erg. Deut. Zentr.-Afr.-Exped., Bot. 2: 146. 1910. TYPE: Vulkan Gebiet: Karisimbi, Stämme von Senecio, ca. 3600 m, *Mildbread 2082* (syntype, presumably at H-Br, not seen); and Ruwenzori: Butagu-Tal, Ulimbi-Berg, an Senecio-Stämmen, 3800 m, *Mildbraed 2652* (syntype, presumably at H-Br, not seen). We have chosen this name to reflect the diagnostic character mentioned by Potier de la Varde (1942).

Leptodontiopsis comprises three more taxa (Crosby et al., 1999), namely, *L. orientalis* Dix. from Borneo and two other African species, *L. macrocarpa* Dix. and *L. elata* Dix. *Leptodontiopsis elata* was distinguished from *L. fragilifolia* by the mostly entire and flexuose-crisped leaves and much taller plants. The species differs furthermore by the absence of conspicuous papillae. The cells can, however, not be characterized as smooth. In transverse section they appear scabrid, with low, inconspicuous thickenings. The other African species, *L. macrocarpa*, is diagnosed solely by the larger capsule. The Asian *L. orientalis* is similar to *L. fragilifolia* but distinguished by Dixon by the squarrose, recurved, and finely acuminate leaves, with orange rows of basal cells. The taxonomic status of these taxa is in need of study. The species share a robust gametophyte to 12 cm high and except for *L. elata*, whose capsules are unknown, long seta, and smooth and gymnostomous capsules. For now, we accept them as distinct, albeit within the genus *Zygodon*. The following new combinations are necessary:

Zygodon elatum (Dix.) Goffinet, comb. nov. *Leptodontiopsis elata* Dix. Smithsonian Misc. Collect. 69: 13. 1918. TYPE: Kenya. Mount Kenya, giant heath zone, 3630 m, 1909–1910, *E. A. Mearns 1557*, (syntypes, BM!, US not seen).

Zygodon macrocarpum (Dix.) Goffinet, comb. nov. *Leptodontiopsis macrocarpa* Dix. J. Bot. 76: 252. 1938. TYPE: Kenya. Aberdare Mts., Naiwasha, Kinangop Forest, 2750 m, epiphytic on tree, 31 Oct. 1934, *G. Taylor 1633* (holotype, BM!).

Zygodon orientalis (Dix.) Goffinet, comb. nov. *Leptodontiopsis orientalis* Dix. J. Linn. Soc., Bot. 50: 91. 1935. TYPE: Borneo. Kinabalu, 14 Nov. 1931, *R. E. Holttum 25668, 25689* (syntypes, presumably at BM, not seen).

D. *MUELLERIELLA*

Based on our phylogenetic inferences we propose to synonymize *Muelleriella* with *Orthotrichum*. The type species of the genus *Muelleriella* (*M. crassifolia* (Hook. f. & Wilson) Dusén, Vitt, 1976) was initially described as a member of *Orthotrichum* (Hooker & Wilson, 1844). Vitt (1976) described two new species in *Muelleriella*, one of which (i.e., *M. engelii* Vitt) had its name later synonymized with a new combination (i.e., *M. atrata* (Mitten) Vitt) based on the earlier name *Orthotrichum atrata* Mitten (Vitt, 1979). Vitt (1976) also treated *Orthotrichum crassifolium* var. *acutum* Müll. Hal. at the subspecific level (*M. crassifolia* subsp. *acuta* (Müll. Hal.) Vitt). Thus, two new combinations are needed to complete the transfer of *Muelleriella* to *Orthotrichum*, and retain the taxonomic concepts of Vitt (1979). Note that since *Muelleriella* appears closely related to a group of cryptoporous *Orthotrichum*, including the type of the genus (*O. anomalum* Hedw.), future decisions to split the genus based on stomatal architecture should not affect the new combination proposed here:

Orthotrichum aucklandicum (Vitt) Goffinet, comb. nov. *Muelleriella aucklandica* Vitt, J.

Hattori Bot. Lab. 40: 94. 1976. TYPE: Auckland Islands. On rocks at water level on NE shore of Lake Hinemoa, 25 m, Musgrave Inlet, 19 Dec. 1972, *D. H. Vitt 9172* (holotype, ALTA!; isotypes, CHR not seen, MSC not seen, NY not seen).

Orthotrichum crassifolium subsp. **acutum** (Müll. Hal.) Goffinet, comb. nov. *Orthotrichum crassifolium* var. *acutum* Müll. Hal., Syn. 1: 699. 1849. *Muelleriella crassifolia* subsp. *acuta* (Müll. Hal.) Vitt, J. Hattori Bot. Lab. 40: 103. 1979. TYPE: Kerguelen Island. Antarctic Expedition 1839–1843, *J. D. Hooker s.n.* (lectotype, designated by Vitt (1976: 103, Hooker BM, not seen).

Conclusions

The Orthotrichioideae sensu Goffinet and Vitt (1998) comprised several monospecific or species-poor genera. Phylogenetic inferences based on four loci suggest that the Orthotrichoideae are polyphyletic (i.e., *O.* subg. *Exiguifolium* belongs to *Leratia*) and that the Zygodonteae are paraphyletic (i.e., *Bryomaltaea* and *Leratia* compose a lineage sister to the Macromitrioideae). The speciose genera *Zygodon* and *Orthotrichum* are also para- or polyphyletic. *Leptodontiopsis*, a genus of four species confined to high altitudes in the Old World tropics, is nested and thus synonymized with *Zygodon*. Similarly, *Muelleriella* and *Orthomitrium* represent derived lineages within the genus *Orthotrichum*. The bispecific *Pentastichella*, monospecific *Pleurorthotrichum* (Zygodonteae) and *Sehnemobryum*, and the bispecific *Stoneobryum* (Orthotricheae) are maintained as distinct genera. *Orthotrichum* subg. *Orthophyllum* is best treated as a genus distinct from *Orthotrichum* (i.e., *Nyholmiella*). The monophyly of *Orthotrichum* remains ambiguous due to the possible close affinities of *Ulota* to those species of *Orthotrichum* with superficial stomata.

Further studies are needed to elucidate the possible hybrid origin of *Sehnemobryum* and *Stoneobryum*, the relationship of *Pleurorthotrichum* and *Pentastichella* to *Zygodon* s. str., and the common ancestry of *Ulota* and phaneroporous *Orthotrichum*.

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SYSTEMATIC STUDIES OF NON-HYPNANAEAN PLEUROCARPS: ESTABLISHING A PHYLOGENETIC FRAMEWORK FOR INVESTIGATING THE ORIGINS OF PLEUROCARPY

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Abstract

Phylogenetic relationships among and within non-hypnanaean pleurocarpous moss groups and putatively related bryalean acrocarps were investigated by cladistic analysis of chloroplast (*rbc*L, *rps*4) and mitochondrial (*nad*5) nucleotide sequence data under both parsimony and likelihood criteria. Particular emphasis was placed on the Southern Hemisphere family Rhizogoniaceae. The Rhizogoniaceae are shown to be polyphyletic and to comprise the major part of a grade of protopleurocarps and acrocarps basal to a derived pleurocarpous clade that consists of two well-supported sister groups, the Hypnanae and a monophyletic assemblage of predominantly "eubryalean" pleurocarps (the Hypnodendraceae, Spiridentaceae, Cyrtopodaceae, Racopilaceae, and Pterobryellaceae). The rhizogoniaceous grade includes *Aulacomnium, Orthodontium lineare,* and *Calomnion* and appears to comprise three clades, although two of these are weakly supported. The sister group to the apical pleurocarpous clade is likely to be either *Pyrrhobryum* sect. *Pyrrhobryum* (excluding *P. dozyanum*) or a clade consisting of these taxa in addition to *Mesochaete* and *Aulacomnium.* Although relationships between some of the major groups within the rhizogoniaceous grade remain poorly supported, a phylogenetic hypothesis is suggested that can inform and constrain the process of modeling the evolution of the pleurocarpous condition.

Key words: bryophyte, Hypnodendraceae, mitochondrial, moss, *nad*5, phylogeny, pleurocarpy, *rbc*L, *rps*4, Rhizogoniaceae, systematics.

Within the diplolepidous-alternate mosses, one of the most important remaining phylogenetic questions is the precise circumscription of the speciose and diverse pleurocarp group. Intrinsically linked with this is the attempt to understand how pleurocarpy evolved and the manner in which its several defining features are related.

Traditionally, the vast majority of the pleurocarps have been classified in three orders, the Hypnales (M. Fleisch.) W. R. Buck & Vitt, Leucodontales (M. Fleisch.) W. R. Buck & Vitt, and Hookeriales (M. Fleisch.) M. Fleisch. While circumscription of these has varied, it is now generally accepted that together they form a clade (De Luna et al., 1999; Buck et al., 2000a, b; Tsubota et al., 2002; Newton & Cox, unpublished data), although the Hypnales and the Leucodontales themselves are probably not monophyletic and the positions of morphologically isolated groups, such as the Hypopterygiaceae and the ptychomnioid/garovaglioid mosses, remain unclear. Buck et al. (2000a) defined the subclass Hypnidae W. R. Buck, Goffinet & A. J. Shaw as encompassing these three orders (subsequently superorder Hypnanae sensu Buck & Goffinet, 2000) and proposed merging the Hypnales with the Leucodontales as the Hypnales s.l. In the case of this large clade, it is relatively simple to conceive of pleurocarpy in the broad sense as a suite of characters that, when occurring together, adapts a moss towards a creeping or weft-forming habit that allows uninterrupted gametophytic growth simultaneous with the production of sporophytes. Outside of the Hypnanae, however, there are a few other relatively small families in which pleurocarpy also occurs, although often in unusual forms and without associated secondary pleurocarpous traits. Recent phy-

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logenetic analyses resolve various exemplars from these taxa (together or in isolation) as sister to the other pleurocarps (De Luna et al., 1999; Buck et al., 2000a; De Luna et al., 2000; Tsubota et al., 2002). By far the most morphologically diverse of these groups is the Rhizogoniaceae, a rather loose assemblage of genera traditionally placed in the predominantly acrocarpous order Bryales and having an almost exclusively Southern Hemisphere distribution associated with the fragments of Cretaceous Gondwana.

Together with a few other smaller families, including the Spiridentaceae, Racopilaceae, Hypnodendraceae, and Cyrtopodaceae, the Rhizogoniaceae form a poorly resolved association of taxa within which most species appear to be pleurocarpous, depending upon the definition of the term that is accepted (see La Farge-England, 1996; Newton & De Luna, 1999. Note that Koponen, 1988, considered all of the Rhizogoniaceae to be acrocarpous). Each of these families is individually well circumscribed and probably monophyletic, with the exception of the Hypnodendraceae (Bell & Newton, 2004) and the Rhizogoniaceae, the latter consisting of eight rather different genera associated to a lesser or greater degree by characters of perichaetial position, leaf morphology, and cell shape. In a recent combined molecular and morphological study based upon *rbc*L and a large morphological data set (De Luna et al., 1999), the single rhizogoniaceous exemplar *Pyrrhobryum spiniforme* was supported as sister to a monophyletic pleurocarpous clade, the latter containing the Hypnanae resolved as sister to a well-supported group of exemplars from the Racopilaceae, Hypnodendraceae, Spiridentaceae, and Cyrtopodaceae. This group of four families (the non-rhizogoniaceous eubryalean pleurocarps, henceforth referred to as the hypnodendroid pleurocarps) is also very well supported in other studies (e.g., Cox et al., 2000; Tsubota et al., 2002), its precise circumscription and phylogeny being the subject of a separate study (Bell & Newton, 2004). The relationships among the Hypnanae, the hypnodendroid pleurocarps, the diverse genera of the Rhizogoniaceae, and the acrocarpous Bryalean families (particularly the Mniaceae and the Bartramiaceae) remain uncertain, however, and are central to understanding the manner in which the suite of characters that defines "hypnanaean pleurocarpy" came into being.

The taxonomic history of the eubryalean pleurocarps since Brotherus (1924, 1925a–c) is one of relative stability at higher levels combined with some vacillation in the placement of several families. In the second edition of *Die natürlichen Pflanzenfamilien*, Brotherus (1924) classified the Rhizogoniaceae in the order Eubryales, a predominantly acrocarpous group that included the Mniaceae, Bartramiaceae, and Aulacomniaceae, but also the pleurocarpous Hypnodendraceae and Spiridentaceae. The remainder of the pleurocarps were placed in the orders Isobryales, Hookeriales, and Hypnobryales (Brotherus, 1925a–c), broadly corresponding to the later Leucodontales, Hookeriales, and Hypnales, respectively (Buck & Vitt, 1986). The perception of the Cyrtopodaceae and Racopilaceae as distinct from the eubryalean pleurocarps is indicated by their placement in the Isobryales. This higher level phylogenetic framework altered little during the next half century, with Vitt (1984) recognizing Brotherus's orders at the subordinal level and maintaining the positions of the taxa under consideration, with the exception of the Racopilaceae, which he moved to the Hypnineae (i.e., Hypnobryales). Buck and Vitt's (1986) classification only two years later, however, was in many ways at odds with others both before and since with regard to the placement of the basal pleurocarpous families. The bryalean pleurocarps were classified in the suborder Rhizogoniineae and included the Rhizogoniaceae, Spiridentaceae, Racopilaceae, Hypopterygiaceae, and Helicophyllaceae, although not the Cyrtopodaceae (placed in the Leucodontales) or even the Hypnodendraceae, which was moved to the Hypnales. The Hypopterygiaceae and Helicophyllaceae, seen as closely related to the Racopilaceae by Buck and Vitt (all three families share a dimorphous, complanate leaf arrangement), are now placed in the Hookeriales and Hedwigiales, respectively (Buck & Goffinet, 2000). In the current generally accepted higher level classification of mosses (Buck & Goffinet, 2000), the Hypnodendraceae, Rhizogoniaceae, Cyrtopodaceae, Spiridentaceae, and Racopilaceae are placed in a separate superorder, the Rhizogonianae (containing the single order Rhizogoniales) along with the Pterobryellaceae and the acrocarpous families Calomniaceae and Mitteniaceae. This group effectively lumps the hypnodendroid pleurocarps together with the Rhizogoniaceae, Calomniaceae, and Mitteniaceae.

Support for a monophyletic Rhizogoniales is unconvincing and generally derived from analyses that have used *rbc*L data exclusively. Withey (1996a, b) recovered a clade (her Clade A) using *rbc*L containing exemplars from the Hypnodendraceae, Spiridentaceae, Racopilaceae, Cyrtopodaceae, and Rhizogoniaceae, although the acrocarpous *Plagiomnium* T. J. Kop., *Bartramia* Hedw., and *Philonotis* Brid. were also included and the group was effectively unsupported, with a bootstrap

percentage of 54 and DI of 2. De Luna et al. (1999) resolved a very similar clade in their analysis using *rbc*L data alone, although again the group was poorly supported, and it was not recovered in their combined analysis of *rbc*L and morphological data, in which *Pyrrhobryum* Mitt. was placed as sister to a clade of hypnodendroid pleurocarps and hypnanaean pleurocarps. Recently, Tsubota et al. (2002) obtained a 91% LBP (local bootstrap probability, Adachi & Hasegawa, 1996) on a maximum likelihood tree for a clade comprising three exemplars from the Cyrtopodaceae in addition to *Hypnodendron menziesii*, *Racopilum convolutaceum* (Müll. Hal.) Reichardt, *Pyrrhobryum vallis-gratiae*, *Aulacomnium turgidum*, and *Orthodontium lineare*; this analysis also used *rbc*L exclusively.

Other recent analyses of the basal pleurocarps (the combined analysis of De Luna et al., 1999; O'Brien, in review; Newton & Cox, unpublished data) suggest that the Rhizogoniales is paraphyletic. The only molecular study to sample extensively from the Rhizogoniaceae was that of O'Brien (in review), who was primarily interested in the position of the Aulacomniaceae. Using the chloroplast *rps*4, *rbc*L, *atp*B, and *trn*L regions, O'Brien resolved the Rhizogoniaceae as a grade basal to a clade representing the rest of the pleurocarps, within which six exemplars from the hypnodendroid pleurocarps were sister to the Hypnanae. Exemplars from the Mniaceae were sister to (the rhizogoniaceous grade + (Hypnanae + hypnodendroid pleurocarps)), with the Rhizogoniaceae resolved in three separate clades. An *Aulacomnium* Schwägr./*Mesochaete* Lindb./*Hymenodontopsis* Herzog/*Pyrrhobryum* sect. *Bifariella* (Müll. Hal.) Manuel clade was sister to the (Hypnanae + hypnodendroid pleurocarps), with *Aulacomnium* sister to *Mesochaete*. *Leptotheca* Schwägr. was resolved in a clade with *Hymenodon* Hook. f. & Wilson, the remainder of the family occurring in a large third clade, within which *Pyrrhobryum* sect. *Pyrrhobryum* grouped with *Cryptopodium* Brid. and *Calomnion* Hook. f. & Wilson. The latter was, in turn, resolved as sister to a *Rhizogonium* Brid./*Goniobryum* Lindb. group. Although O'Brien's phylogeny is well resolved, sampling from several Rhizogoniaceous genera is limited and support provided only in the form of decay indices, which are low for several important nodes.

Existing evidence, therefore, suggests that the Rhizogoniaceae may represent a grade at the base of the other pleurocarps within which characters normally closely associated with pleurocarpy are intermittently present, present in unusual forms, or present in unique combinations. This is consistent with the hypothesis that the family contains the only extant members of the lineage or lineages within which pleurocarpy first evolved. By establishing a robust phylogeny for the group and resolving relationships with the Hypnanae, the hypnodendroid pleurocarps, and the acrocarpous bryalean taxa, a framework will be constructed on which the development of pleurocarpy can potentially be mapped. This could, in turn, provide invaluable insights into the nature of the adaptations that have permitted the dramatic radiation of the Hypnanae.

The aim of this study is to develop a credible hypothesis of phylogenetic relationships between the principal clades of the pleurocarpous and protopleurocarpous mosses (i.e., the Hypnanae, the hypnodendroid pleurocarps, and the genera of the Rhizogoniaceae) and potentially related acrocarpous taxa. Corroboration of the monophyly of these groups will also be sought, particularly among the Rhizogoniaceae. Although the Rhizogoniaceae contain several putatively monophyletic units at this phylogenetic level, there is currently little evidence to suggest that the family is monophyletic, and hence taxon sampling will be focused on this group. It is likely that taxonomy has actually hindered the understanding of phylogeny within the basal pleurocarps in recent years. The implicit perception of the Rhizogoniaceae as a homogenous group may have encouraged the under-representation of the considerable structural diversity it contains in higher level molecular studies, most of which have used just one or two exemplars from the family (e.g., Cox et al., 2000; De Luna et al., 2000; Tsubota et al., 2002).

A phylogenetic hypothesis will be developed using a two-stage approach. An initial series of analyses based on the parsimony criterion will sample over a broad taxonomic range in considerable detail, using data from the chloroplast *rps*4 and *rbc*L regions. The object will be to establish (or corroborate) the monophyly of critical groups, particularly at the generic and/or family levels, in order to provide a sound basis for more restricted taxon sampling in a second analysis, in which the mitochondrial *nad*5 region will be used in addition to *rps*4 and *rbc*L and the criterion of maximum likelihood will also be applied. Such an approach maximizes the productivity of sampling effort, as although the *nad*5 region is known to provide resolution over a wide phylogenetic range in mosses, including deeper nodes (Beckert et al., 1999; Bell & Newton, 2004), it is relatively difficult and time-consuming to work with and very few sequences for potential outgroup taxa are currently available. The chloro-

plast genes, by contrast, are easy to sequence, far more widely used, and can be expected to satisfactorily resolve clades in mosses at lower phylogenetic levels (Goffinet et al., 2001; Tsubota et al., 2002).

Materials and Methods

TAXON SAMPLING

The data set for the *rps*4 + *rbc*L analysis consisted of 99 taxa, with sequences of both genes obtained for all (Table 1). From the Rhizogoniaceae, 23 species were sampled from seven of the eight genera, representing approximately 70% of the 33 accepted species. Unfortunately, no sufficiently fresh material of the monospecific *Hymenodontopsis* (Rhizogoniaceae) could be obtained. A further 2 species of Rhizogoniaceae (*Pyrrhobryum pungens* and *Rhizogonium lindigii*) and *Mittenia plumula*, for which only *rps*4 sequences were available, were not included in the main analysis, as this would have entailed a considerable increase in the percentage of missing data in the matrix. A separate analysis of the *rps*4 + *rbc*L data set was conducted with these taxa included (see below). In the case of *Pyrrhobryum*, the largest genus in the Rhizogoniaceae, care was taken to sample extensively from both of the sections (*Pyrrhobryum* and *Bifariella*) recognized by Manuel (1980). A representative group of 16 exemplars from the hypnodendroid pleurocarps was included, based partially on the phylogeny obtained for that group by Bell and Newton (2004). The Hypnanae was represented by 21 species, including exemplars from all three traditional orders. Sampling from this group reflected phylogenetic width rather than the diversity of individual clades, hence isolated and putatively "basal" groups, such as the Garovagliaceae, Ptychomniaceae, Hypopterygiaceae, and Hookeriales (Newton & Cox, unpublished data), are disproportionately represented. *Bryobrothera crenulata*, transferred from the Rhizogoniaceae to the Hookeriaceae by Norris and Robinson (1979), was included.

Sampling from the acrocarpous Bryalean taxa was wide ranging, although heavily biased towards groups that have been suggested as being closely related to the Rhizogoniaceae. *Mittenia plumula* was thought to be closely related to *Rhizogonium* by Goffinet et al. (2001) and Buck and Goffinet (2000), although it is likely that the single sequence of *rps*4 on which this hypothesis was based represents a misidentified specimen of *Rhizogonium* (Bell & Newton, 2004). We were able to obtain a sequence of *rps*4 for *Mittenia plumula*, but not *rbc*L or *nad*5. As *rps*4 sequences alone were also obtained for two rhizogoniaceous taxa, *Pyrrhobryum pungens* and *Rhizogonium lindigii*, we decided to analyze the two-gene data set separately with these three taxa included. The function of this separate analysis was to test the phylogenetic positions of these taxa without compromising the validity of the main analysis by the unnecessary introduction of large amounts of missing data.

Two species of *Calomnion*, an acrocarpous genus often suggested as a possible close relative of the Rhizogoniaceae (Brotherus, 1924; Vitt, 1995; O'Brien, in review; McDaniels, pers. comm.), were included in the principal two-gene analysis, as were two species of *Aulacomnium*.

The acrocarpous family most often regarded as a potential close relative of the Rhizogoniaceae is the Mniaceae. Koponen (1988) suggested that *Pyrrhobryum*, *Cryptopodium*, *Hymenodontopsis*, and *Leptotheca* are more closely related to certain genera in the Mniaceae than to the other Rhizogoniaceae, mostly on the basis of a manually derived cladogram utilizing a small set of morphological characters. A novel classification was proposed in which these four taxa were placed in the Mniaceae along with *Mnium* Hedw., *Leucolepis* Lindb., and *Trachycystis* T. J. Kop., leaving only *Rhizogonium*, *Hymenodon*, *Mesochaete*, and *Goniobryum* in the Rhizogoniaceae. A third family, the Plagiomniaceae, was erected to encompass the mniaceous genera *Plagiomnium*, *Orthomnion* Wilson, and *Pseudobryum* (Kindb.) T. J. Kop., while the positions of *Cinclidium* Sw., *Cyrtomnium* Holmen, and *Rhizomnium* (Broth.) T. J. Kop. were left uncertain. If accepted, this hypothesis would necessitate that any phylogenetic study of the Rhizogoniaceae should sample extensively from the Mniaceae. However, in addition to being at odds with more recent hypotheses derived from molecular data (e.g., Cox et al., 2000), there are several serious problems with both the methodology used in this study and a number of the character conceptions and codings. First, the two cladograms on which Koponen's hypotheses of the paraphyly of the Rhizogoniaceae and Mniaceae are based were manually constructed (as was common practice at this time), and neither represents a most parsimonious solution to the matrix. In addition, the trees were rooted on a priori assumptions of plesiomorphy made for each of the binary characters. PAUP* finds 23 MPTs of length 20 from the matrix used to produce Koponen's trees (2 steps less than his fig. 2 and 1 step less than fig. 3). When rooted on the same assumptions of plesiomorphy, the strict consensus tree retains ((*Rhizogonium*, *Hymenodon*, *Leptotheca*, *Hymenodontopsis*,

Table 1. Taxa sampled. Sequences generated directly for this study are in bold with voucher information. Previously published sequences are referenced by GenBank accession numbers. Circumscription of families follows Buck and Goffinet (2000), other than for the positions of *Braithwaitea*, which follows Touw (1971), and *Cyathophorum*, which follows Kruijer (2002).

Taxon	Voucher	GenBank accession No.		
		*rps*4	*rbc*L	*nad*5
Adelotheciaceae W. R. Buck				
Bryobrothera crenulata (Broth. & Paris) Thér.	**Australia, *Bell 435* (BM)**	**AY631139**	**AY631175**	**AY631209**
Aulacomniaceae Schimp.				
Aulacomnium androgynum (Hedw.) Schwägr.	**U.K., *Bell 1299* (BM)**		**AY631174**	
		AF023811		AJ291564
Aulacomnium turgidum (Wahlenb.) Schwägr.		AF023809	AJ275180	—
Bartramiaceae Schwägr.				
Bartramia halleriana Hedw.		AF265358	AF231090	Z98961
Bartramia mathewsii Mitt.		AF491026	AF491017	—
Breutelia arundinifolia (Duby) M. Fleisch.		AF491040	AF491006	—
Breutelia chrysea (Müll. Hal.) A. Jaeger		AF491030	AF491023	—
Breutelia polygastrica (Müll. Hal.) Broth.		AF491027	AF491025	—
Fleischerobryum longicolle (Hampe) Loeske		AF491038	AF491021	—
Leiomela bartramioides (Hook.) Paris		AF478284	AF478238	—
Philonotis fontana (Hedw.) Brid.	**U.K., *Bell 1289* (BM)**		**AY631192**	**AY631223**
		AF023801		
Philonotis thwaitesii Mitt.		AF491034	AF491019	—
Quathlamba debilicostata Magill		AF491032	AF491008	—
Bryaceae Schwägr.				
Anomobryum julaceum (Schrad. ex G. Gaertn., B. Mey. & Scherb.) Schimp.		AF023786	AJ275172	—
Bryum alpinum Huds. ex With.	**U.K., *Bell 789* (BM)**		**AY631176**	**AY631210**
		AF023783		
Calomniaceae Kindb.				
Calomnion brownseyi Vitt & H. A. Mill.	**New Zealand, *Beveridge s.n.* (BM)**	**AY631140**	**AY631177**	**AY631211**
Calomnion complanatum (Hook. f. & Wilson) Lindb.	**Australia, *Bell 600* (BM)**	**AY631141**	**AY631178**	**AY631212**
Cyrtopodaceae M. Fleisch.				
Bescherellia elegantissima Duby	**New Caledonia, *Bell 364* (BM)**	**AY524472**	**AY524444**	**AY608578–AY608579**
Cyrtopus setosus (Hedw.) Hook. f.	**New Zealand, *Beveridge s.n.* (BM)**	**AY524479**	**AY524451**	**AY608582–AY608583**

Table 1. Continued.

Taxon	Voucher	GenBank accession No. *rps*4	*rbc*L	*nad*5
Dicranaceae Schimp.				
Dicranum scoparium Hedw.		AF231277	AF231067	Z98956
Encalyptaceae Schimp.				
Bryobrittonia longipes (Mitt.) D. G. Horton		AF023778	AJ275168	—
Encalypta rhaptocarpa Schwägr.		AF023777	J275167	—
Fontinalaceae Schimp.				
Fontinalis antipyretica Hedw.		AF023817	AJ275183	AJ291570
Funariaceae Schwägr.				
Funaria hygrometrica Hedw.		AF023776	AF005513	Z98959
Garovagliaceae (M. Fleisch.) W. R. Buck & Vitt.				
Euptychium cuspidatum (Mitt.) Mitt.	**New Caledonia, *Newton 5373* (BM)**	**AY631144**	**AY631180**	—
Garovaglia elegans (Dozy & Molk.) Hampe ex Bosch & Sande Lac.	**Australia, *Newton 5463* (BM)**	**AY631145**	**AY631181**	**AY631215**
Garovaglia subelegans Broth.	**Papua New Guinea, *Baker 740* (RNG)**	**AY631146**	**AY631182**	—
Hedwigiaceae Schimp.				
Hedwigia ciliata (Hedw.) P. Beauv.		AJ251309	AF231073	Z98966
Hookeriaceae Schimp.				
Hookeria acutifolia Hook. & Grev.		AF143071	AF158170	—
Hookeria lucens (Hedw.) Sm.	**U.K. *Cox 118* (RNG)**	AJ251316	**AY631185**	Z98969
Hylocomiaceae (Broth.) M. Fleisch.				
Rhytiadelphus squarrosus (Hedw.) Warnst.		AF143033	AB024667	—
Rhytidiadelphus triquetrus (Hedw.) Warnst.	**U.K., *Bell 798* (BM)**	**AF524464**	**AY524436**	Z98971
Hypnaceae Schimp.				
Hypnum lindbergii Mitt.		AF143035	AB029390	—
Hypnodendraceae Broth.				
Braithwaitea sulcata (Hook.) A. Jaeger & Sauerb.	**Australia, *Newton 5538* (BM)**	**AY524469**	**AY524441**	**AY524524**
Hypnodendron auricomum Broth. & Geh.	**Papua New Guinea, *Eddy 2077* (BM)**	**AY524487**	**AY524459**	—

Table 1. Continued.

Taxon	Voucher	GenBank accession No.		
		*rps*4	*rbc*L	*nad*5
Hypnodendron comosum (Labill.) Mitt.	**Australia, *Bell 648* (BM)**	**AY524466**	**AY524438**	**AY524521**
Hypnodendron dendroides (Brid.) Touw	**Malaysia, *Gardner 1* (BM)**	**AY524482**	**AY524454**	**AY524537**
Hypnodendron diversifolium Broth. & Geh.	**Malaysia, *Gardner 2* (BM)**	**AY524480**	**AY524452**	**AY524535**
Hypnodendron menziesii (Hook.) Paris	**New Caledonia, *Bell 376* (BM)**	**AY524467**	**AY524439**	**AY524522**
Hypnodendron spininervium (Hook.) A. Jaeger & Sauerb.	**Australia, *Bell 601* (BM)**	**AY524481**	**AY524453**	**AY524536**
Hypnodendron subspininervium (Müll. Hal.) A. Jaeger	**Malaysia, *Ellis 380* (BM)**	**AY532393**	**AY532391**	**AY524538**
Hypopterygiaceae Mitt.				
Cyathophorum bulbosum (Hedw.) Müll. Hal.	**Australia, *Bell 476* (BM)**	**AY631143**	**AY631179**	**AY631214**
Hypopterygium tamarisci (Sw.) Brid. ex Müll. Hal.		AF143077	AF232695	—
Lopidium concinnum (Hook.) Wilson	**Australia, *Newton 5654* (BM)**	**AY631153**	**AY631190**	**AY631221**
Leptostomataceae Schwägr.				
Leptostomum macrocarpum (Hedw.) Bach. Pyl.		AF023790	AJ275178	—
Leucodontaceae Schimp.				
Leucodon julaceus (Hedw.) Sull.	**Mexico, *De Luna 2164* (XAL)**	**AY631152**	AF231075	—
Pterogonium gracile (Hedw.) Sm.	**U.S.A., *Newton 5202* (BM)**	**AY631157**	**AY631194**	Z98968
Meesiaceae Schimp.				
Amblyodon dealbatus (Sw. ex Hedw.) Bruch & Schimp.		AF023806	AJ275181	—
Leptobryum pyriforme (Hedw.) Wilson		AF023802	AF231072	—
Meesia uliginosa Hedw.		AF306994	AJ275182	—
Meteoriaceae Kindb.				
Ancistrodes genuflexa (Müll. Hal.) Crosby	**Chile, *Bell 1253* (BM)**	**AY631138**	**AY631173**	—
Mitteniaceae Broth.				
Mittenia plumula (Mitt.) Lindb.	**Australia, *Stone s.n.* (BM)**	**AY631155**	—	—
Mniaceae Schwägr.				
Leucolepis menziesii (Hook.) Steere		AJ251312	AJ275177	—
Mielichhoferia bryoides (Harv.) Wijk & Margad.		AF023794	AJ275165	—
Mnium hornum Hedw.		AF023796	AF226820	AJ291567
Plagiomnium cuspidatum (Hedw.) T. J. Kop.		AF491033	—	Z98965
Plagiomnium japonicum (Lindb.) T. J. Kop.		—	AB050992	—

Table 1. Continued.

Taxon	Voucher	GenBank accession No. *rps*4	*rbc*L	*nad*5
Pohlia cruda (Hedw.) Lindb.		AF023795	AJ275175	—
Pohlia nutans (Hedw.) Lindb.	**Canada, *Hedderson 10481* (RNG)**	**AY631156**	**AY631193**	AJ291565
Rhizomnium punctatum (Hedw.) T. J. Kop.		AF478290	AF478237	—
Neckeraceae Schimp.				
Thamnobryum alopecurum (Hedw.) Nieuwl. ex Gangulee	**U.K., *Cox 147* (RNG)**	AF023834	**AY532392**	AJ291571
Orthodontiaceae (Broth.) Goffinet				
Orthodontium lineare Schwägr.		AF023800	AJ275174	AJ291566
Orthotrichaceae Arn.				
Orthotrichum lyellii Hook. & Taylor		AF023814	AF005536	—
Ulota crispa (Hedw.) Brid.	**France, *James s.n.* (BM)**	AF306972	**AY631208**	AJ291568
Zygodon intermedius Bruch & Schimp.		AF223061	AF005532	—
Pottiaceae Schimp.				
Tortula ruralis (Hedw.) G. Gaertn., B. Mey. & Scherb.		AF023831	AJ275169	—
Pterobryellaceae (Broth.) W. R. Buck & Vitt				
Pterobryella praenitens Müll. Hal.	**Australia, *Streimann 55850* (CBG)**	**AY524483**	**AY524455**	**AY524539**
Ptychomitriaceae Schimp.				
Ptychomitrium gardneri Lesq.		AF023779	AF005549	—
Ptychomniaceae M. Fleisch.				
Glyphothecium sciuroides (Hook.) Broth.	**Australia, *Newton 5694* (BM)**	**AY631147**	**AY631183**	**AY631216**
Hampeella alaris (Dixon & Sainsbury) Sainsbury	**Australia, *Newton 5682* (BM)**	**AY524463**	**AY524435**	**AY524519**
Ptychomnion aciculare (Brid.) Mitt.	**Australia, *Streimann 38061* (DUKE)**	**AY631158**	AF233576	—
Racopilaceae Kindb.				
Powellia involutifolia Mitt.	**Australia, *Newton 5431* (BM)**	**AY524465**	**AY524437**	**AY524520**
Racopilum spectabile Reinw. & Hornsch.	**New Caledonia, *Newton 5351* (BM)**	**AY524478**	**AY524450**	**AY524533**
Rhacocarpaceae Kindb.				
Rhacocarpus purpurascens (Brid.) Paris		AF023815	AJ275171	Z98967

Table 1. Continued.

Taxon	Voucher	GenBank accession No. *rps*4	*rbc*L	*nad*5
Rhizogoniaceae Broth.				
Cryptopodium bartramioides (Hook.) Brid.	**New Zealand, *Fife 11494* (BM)**	**AY631142**		**AY631213**
			AF231084	
Goniobryum subbasilare (Hook.) Lindb.	**Australia, *Newton 5797* (BM)**	**AY631148**	**AY631184**	**AY631217**
Hymenodon pilifer Hook. f. & Wilson	**Australia, *Bell 596* (BM)**	**AY631149**	**AY631186**	**AY631218**
Hymenodon sphaerothecius Besch.	**New Caledonia, *Bell 363* (BM)**	**AY631150**	**AY631187**	**AY631219**
Leptotheca boliviana Herzog	**Colombia, *Churchill 16400* (H)**		**AY631188**	—
		AF023816		
Leptotheca gaudichaudii Schwägr.	**Australia, *Bell 696* (BM)**	**AY631151**	**AY631189**	**AY631220**
Mesochaete taxiforme (Hampe) Watts & Whitel.	**Australia, *Bell 464* (BM)**	**AY524462**	**AY524434**	**AY524518**
Mesochaete undulata Lindb.	**Australia, *Bell 500* (BM)**	**AY631154**	**AY631191**	**AY631222**
Pyrrhobryum bifarium (Hook.) Manuel	**Australia, *Bell 770* (BM)**	**AY631159**	**AY631195**	**AY631224**
Pyrrhobryum dozyanum (Sande Lac.) Manuel	**Taiwan, *Huttunen 1425* (H)**	**AY631160**	**AY631196**	**AY631225**
Pyrrhobryum latifolium (Bosch & Sande Lac.) Mitt.	**Taiwan, *Shevock 14457* (MO)**	**AY631161**	**AY631197**	—
Pyrrhobryum medium (Besch.) Manuel	**New Caledonia, *Bell 349* (BM)**	**AY631162**	**AY631198**	**AY631226**
Pyrrhobryum mnioides (Hook.) Manuel	**Australia, *Bell 652* (BM)**	**AY631163**	**AY631199**	**AY631227**
Pyrrhobryum novae-caledoniae (Besch.) Manuel	**New Caledonia, *Bell 409* (BM)**	**AY631164**	**AY631200**	**AY631228**
Pyrrhobryum paramattense (Müll. Hal.) Manuel	**Australia, *Bell 301* (BM)**	**AY631165**	**AY631201**	**AY631229**
Pyrrhobryum pungens (Sull.) Mitt.	**U.S.A., *Newton 2740* (BM)**	**AY631166**	—	—
Pyrrhobryum spiniforme (Hedw.) Mitt.	**Mexico, *Newton 5186A* (BM)**	**AY524485**	**AY524457**	**AY524541**
Pyrrhobryum vallis-gratiae (Hampe in Müll. Hal.) Manuel	**S. Africa, *Hedderson 13347* (BM)**	**AY631167**	**AY631202**	**AY631230**
Rhizogonium distichum (Sw.) Brid.	**Australia, *Bell 310* (BM)**	**AY524461**	**AY524433**	
	Australia, *Bell 530* (BM)			**AY52517**
Rhizogonium graeffeanum (Müll. Hal.) A. Jaeger	**Australia, *Bell 429* (BM)**	**AY631168**	**AY631203**	**AY631231**
Rhizogonium lindigii (Hampe) Mitt.		AF023826	—	—
Rhizogonium novae-hollandiae (Brid.) Brid.	**Australia, *Bell 633* (BM)**	**AY631169**	**AY631204**	**AY631232**
Rhizogonium pennatum Hook. f. & Wilson	**Australia, *Bell 720* (BM)**	**AY631170**	**AY631205**	**AY631233**
Rutenbergiaceae (Broth.) M. Fleisch.				
Rutenbergia madagassa Geh. & Hampe	**Madagascar, *Fisher 33* (BM)**	**AY524486**	**AY524458**	**AY524542**
Schistostegaceae Schimp.				
Schistostega pennata (Hedw.) F. Weber & D. Mohr	**Germany, *Newton 4997* (BM)**	**AY631171**	**AY631206**	
				AJ224856

Table 1. Continued.

Taxon	Voucher	GenBank accession No.		
		*rps*4	*rbc*L	*nad*5
Spiridentaceae Kindb.				
Spiridens camusii Thér.	**New Caledonia, *Bell 416* (BM)**	**AY524475**	**AY524447**	**AY524530**
Splachnaceae Grev. & Arn.				
Tayloria lingulata (Dicks.) Lindb.		AF023807	AF005516	—
Timmiaceae Schimp.				
Timmia sibirica Lindb. & Arnell		AF023775	AJ275166	—
Trachylomataceae (M. Fleisch.) W. R. Buck & Vitt				
Trachyloma planifolium (Hedw.) Brid.	**Australia, *Streimann 7261* (RNG)**	**AY631172**	**AY631207**	—
Wardiaceae W. H. Welch				
Wardia hygrometrica Harv. & Hook.		AF023782	AJ275170	—

Pyrrhobryum, Cryptopodium, (Trachycystis, Leucolepis)) Mnium, (Goniobryum, Mesochaete)). This is consistent with Koponen's figure 3, but not with figure 2, his preferred phylogeny. Only the grouping of *Goniobryum* with *Mesochaete* has a bootstrap value above 60 (based only on the dubiously coded character 3 (see below)), and of the 15 characters, 7 are autapomorphies, leaving only 8 phylogenetically informative characters for 11 taxa. Thus, neither of Koponen's trees is a supportable phylogenetic hypothesis unless an appeal is made to the special significance of a very few informative characters. Furthermore, the coding of several of these is dubious. Character 3, for example, relates to archegonial position, whether "lateral on stems" (coded for *Goniobryum* and *Mesochaete* only) or "terminal on short branches" (Koponen, 1988: 40). All mosses, in fact, have perichaetia terminal on shoots or branches, although those in which fertile branches are extremely short have traditionally been described as having them lateral on stems (see La Farge-England, 1996; Newton & De Luna, 1999). Such highly reduced perichaetial modules are found in the Hypnanae and also in most of the Rhizogoniaceae. A distinction could perhaps be made between taxa, such as *Rhizogonium* and *Pyrrhobryum* sect. *Pyrrhobryum,* in which perichaetial branches are always basal and sometimes produce subperichaetial innovations, and others, such as *Goniobryum* and *Mesochaete,* in which they are more or less distal on stems and do not produce innovations, although arguably this would confound two independent characters. Also, on this basis at least *Pyrrhobryum* sect. *Bifariella* and *Hymenodontopsis* would share a character state (i.e., perichaetial modules distal on stems and without subperichaetial innovations) with *Goniobryum* and *Mesochaete.* Singling out of the latter two genera is, therefore, not justified, as they are not the only taxa within the Rhizogoniaceae with perichaetia lateral on stems, however, this state is interpreted. Given the lack of support for Koponen's hypothesis and the contradictory evidence from molecular studies, it was considered sufficient to include four carefully selected taxa from the Mniaceae in this study, enough to corroborate the monophyly of the family and to represent it as a phylogenetic unit when examining relationships with other taxa.

The Bartramiaceae share some features with the Rhizogoniaceae, and a close relationship between the families has occasionally been suggested (e.g., Griffin & Buck, 1989). The possible placement of *Aulacomnium,* often seen as close to the Bartramiaceae (e.g., Vitt, 1984), within a rhizogoniaceous grade (O'Brien, in review) further supports this. In

addition, pilot analyses resolved exemplars from the Bartramiaceae close to the Rhizogoniaceae. Testing the monophyly of the two families with respect to each other was thus considered a priority, and the availability of sequences of *rps*4 and *rbc*L from recent work on the Bartramiaceae by Virtanen (2003) facilitated this. Ten bartramiaceous exemplars were included in the analysis.

A small number of species from non-bryalean diplolepidous taxa, including the Splachnaceae, Orthotrichaceae, and Hedwigiales, were included, as relationships between the acrocarpous bryalean taxa and other arthrodontous mosses remain uncertain (Cox & Hedderson, 1999; Cox et al., 2000).

In the combined *rps*4, *rbc*L, and *nad*5 matrix, the included taxa were a subset of those sampled for the first analysis (see Table 1), with the exception of *Rutenbergia madagassa*, which was not included in the *rps*4 + *rbc*L matrix. The selection strategy was to include as many Rhizogoniaceae as possible and to represent large, monophyletic, non-rhizogoniaceous groups by smaller numbers of appropriately chosen exemplars (see results). Of the members of the Rhizogoniaceae included in the first matrix, only *Pyrrhobryum latifolium* and *Leptotheca boliviana* were excluded from the three-gene analysis (on the grounds that *nad*5 sequences could not be obtained).

Outgroups for the two-gene analysis were *Timmia sibirica*, *Bryobrittonia longipes*, *Encalypta rhaptocarpa*, five haplolepidous mosses, and the diplolepidous-opposite *Funaria hygrometrica*. In the three-gene analysis two of the haplolepidous mosses (*Dicranum scoparium* and *Schistostega pennata*) and *Funaria hygrometrica* were retained as outgroups. All trees were rooted on *Funaria hygrometrica*.

DNA EXTRACTION AND AMPLIFICATION

Sequences for all applicable genomic regions were obtained for all taxa in each of the two data matrices (Table 1). Sequences for different regions were all from the same species (and where possible from the same specimen), with the exception of *Plagiomnium*, for which *rps*4 and *nad*5 sequences from *P. cuspidatum* were combined with a sequence of *rbc*L from *P. japonicum*. These taxa are closely related to each other (both are placed in the same section of the genus by Koponen, 1968), although clearly it would have been preferable to have obtained all three sequences from a single species.

Total genomic DNA was extracted using a variant of the CTAB protocol (Rogers & Bendich, 1994) and cleaned with the Wizard DNA Clean-up Kit (Promega, Madison, Wisconsin, U.S.A.). PCR reactions were prepared in 100 μl volumes with 10% 10× NH4 reaction buffer (Bioline, London, U.K.), 2 mM $MgCl_2$, 0.3 μM of each primer, 200 μM dNTPs, 0.09% BSA and 2.5 units of Biotaq *Taq* polymerase (Bioline). Thirty or 35 cycles of denaturing, annealing, and extension were performed preceded by an initial melting step of 4 min. at 94°C and a final extension period of 7 min. at 72°C according to the following protocols. *trn*L-F—94°C (30 sec.), 50°C (30 sec.), 72°C (1 min.). *rps*4—94°C (30 sec.), 50°C (30 sec.), 72°C (1 min. 30 sec.). *rbc*L—94°C (30 sec.), 48° (30 sec.), 72°C (1 min.). *nad*5—94°C (30 sec.), 52°C (30 sec.), 72°C (2 min.). The sequences of all primers used have been published in previous studies and were as follows. *Rbc*L—M636, M745R, M1390R (Lewis et al., 1997), NM34 (Cox et al., 2000). *trn*L-F—TRNC, TRNF (Taberlet et al., 1991). *rps*4—RPS5 (Nadot et al., 1994), TRNAS (Cox et al., 2000). *nad*5—K, L, Li, Ki (Beckert et al., 1999). Single double-stranded templates were produced for *trn*L-F and *rps*4, whereas both *rbc*L and *nad*5 were amplified as two overlapping fragments (NM34—M745R and M636—M1390R for *rbc*L, *nad*5K—*nad*5Li and *nad*5L—*nad*5Ki for *nad*5). Fragments were cleaned using either the QIAquick PCR Purification Kit (QIAGEN, Valencia, California, U.S.A.) or the GFX PCR DNA Purification Kit (Amersham Biosciences, Piscataway, New Jersey, U.S.A.) and sequenced using the amplification primers (plus *nad*5Le, Beckert et al., 1999, in the case of *nad*5) and the ABI BigDye Terminator Cycle Sequencing Reaction Kit on an ABI PRISM 377 automated sequencing machine at BM.

SEQUENCE EDITING AND ALIGNMENT

Forward and reverse sequences were edited and assembled into consensus files using SeqMan II and SeqEdit (Lasergene System Software, DNASTAR, Inc.). The *rps*4 and *rbc*L sequences were pasted directly into NEXUS files and aligned by hand while the *nad*5 sequences were first manually aligned using Se-Al v1.d1 (Andrew Rambaut, University of Oxford, ⟨http://evolve.zoo.ox.ac.uk/⟩) before being exported to NEXUS files. Indels were not separately scored due to unresolved theoretical problems regarding homology assessment (e.g., Graham et al., 2000) and in order to allow comparison of analyses conducted under parsimony and likelihood criteria. The aligned matrices are available from the primary author on request.

Table 2. Character sets used in the preliminary study (*rps*4 + *rbc*L, 99 taxa) showing numbers of informative characters and skewness statistic g_1 (Hillis, 1991).

Character set	Total number of characters	Informative characters	% Informative characters	g_1
Combined 2-gene matrix	1834	550	30%	−0.50
*rps*4	556	224	40%	−0.59
*rbc*L	1278	326	26%	−0.34

PHYLOGENY RECONSTRUCTION

Analyses were performed using PAUP* 4.0b10 (Swofford, 2001) running within Windows XP Home Edition on an AMD Athlon XP2400 based PC (2 GHz) with 512MB of RAM. The 3′ intergenic spacer included in the *rps*4 sequences and a few small stretches of the *nad*5 intron could not be confidently aligned and were excluded. Zones at the beginnings and ends of alignments containing significant amounts of missing data were also excluded. Uninformative characters were included in order to allow autapomorphies to contribute to terminal branch lengths in phylograms. All characters were unordered and equally weighted and gaps treated as missing data.

Searches for most parsimonious trees (MPTs) were undertaken using stepwise random taxon addition and TBR branch swapping with branches collapsed when the minimum length was zero. Initial searches of 100,000 replications were performed saving only one tree of length ≥ 1 on each replication. A second search was then performed with no limits on the number of trees saved (nchuck = 0) starting with the trees held in memory from the first search. This search strategy provides a more uniform sampling of tree space within a given time period than the standard method implemented within PAUP* (Swofford, 2001) and is effectively equivalent to the heuristic strategy implemented within Nona (Goloboff, 1998) and Winclada (Nixon, 1999).

A single search for most parsimonious trees was performed on the two-gene data set. A separate analysis on this data set was then performed with *Mittenia plumula*, *Pyrrhobryum pungens*, and *Rhizogonium lindigii* included. For the three-gene data set, searches were performed on the complete matrix, on the *rps*4 and *rbc*L sequences alone (i.e., the chloroplast-derived data), and on the mitochondrial *nad*5 data alone. The partition homogeneity test (Farris et al., 1994, 1995) was performed with 1000 replications each of 500 heuristic searches (nchuck = 1 and chuckscore = 1) on the mitochondrial and chloroplast partitions. Although the value of this test as a criterion for deciding whether data should be combined has been seriously questioned (e.g., Yoder et al., 2001; Barker & Lutzoni, 2002), it nonetheless provides information that may be helpful in comparing the properties of different data sets. Values for the g_1 statistic of skewness in the distribution of the lengths of 30,000 random trees were also obtained for all discrete data compartments (Hillis, 1991).

Bootstrap values for the two-gene data set were obtained using 1000 replications each of 10 full (random addition) standard heuristic searches. For the separate analysis including *Mittenia plumula*, *Rhizogonium lindigii*, and *Pyrrhobryum pungens*, 116 replications of five full heuristic searches were performed, and for the three-gene data set, 1000 replications of 100 searches. DI values were calculated using modified constraint tree files produced by the DNA Stacks Package version 1.3.1. (Eernisse, 1995). Constrained heuristic searches for each node were performed with 1000 initial replications (nchuck = 1 and chuckscore = 1) followed by swapping of trees in memory (nchuck = 0).

A single maximum likelihood search was performed on the three-gene data set, having first established a best-fit model using Modeltest 3.06 (Posada & Crandall, 1998). Models were estimated for all compartments separately (with *nad*5 divided into exon and intron compartments) and the most complex model used in the analysis. One hundred heuristic search replications were performed with random taxon addition.

Results

PRELIMINARY ANALYSIS (*RPS*4 + *RBC*L)

Alignment of the two-gene data set produced a matrix of 1834 characters, of which 550 were informative. Numbers of characters, informative characters, and values of g_1 for all compartments are presented in Table 2.

While all values for g_1 are well within 99% confidence limits for assuming the character sets are significantly more structured than random data (Hillis & Huelsenbeck, 1992), it is notable that the value for *rbc*L is considerably less negative (i.e.,

less significant) than that for *rps*4. While *rbc*L provides a greater total number of informative characters and values for g_1 are known to be related to the size of the data set (Hillis & Huelsenbeck, 1992), this is not in itself sufficient to account for a difference of this magnitude. Hence, we may conclude that the *rps*4 data is more structured than is *rbc*L for these taxa. This region also provides a much greater percentage of informative characters.

Analysis of the matrix under the parsimony criterion resulted in 32 most parsimonious trees (MPTs) of length 3172 (CI = 0.34, CI excluding uninformative characters (CI − U) = 0.28, RI = 0.59). The strict consensus is well resolved, the topology well supported at many apical nodes but effectively unsupported, with decay indices (DI) = 1 and bootstraps < 50%, at most of the deeper ones. The bootstrap consensus tree is shown in Figure 1.

In the strict consensus tree (not shown) a novel topology is recovered in which the Hypnanae are sister to a large clade including the hypnodendroid pleurocarps, a number of rhizogoniaceous groups and a monophyletic (Bartramiaceae + Mniaceae + *Pohlia* Hedw./*Mielichhoferia* Nees & Hornsch.) clade. A group including *Aulacomnium*, *Mesochaete*, and three out of the four exemplars from *Pyrrhobryum* sect. *Bifariella* is sister to the hypnodendroid pleurocarps. All of the higher level nodes implying relationships between the major groups are supported only by DI values of one, however, and as such the overall topology of the trees does not provide a sound basis for a phylogenetic hypothesis (small perturbations of the data set were observed to produce very different topologies). In this context, presentation of the strict consensus would be at best almost meaningless and at worst positively misleading. The bootstrap consensus presented in Figure 1 is the most appropriate diagrammatic summary of the results, given the objectives of the analysis, i.e., the definition of reliably monophyletic groups to inform the sampling strategy for the second analysis.

In the bootstrap consensus (Fig. 1) the ingroup is well supported, the large clade of diplolepidous-alternate mosses resolved with a bootstrap of 96% and a DI of 10. A haplolepidous clade and *Timmia sibirica* are in unresolved positions relative to the ingroup (the combined clade with a bootstrap of 68% and DI of 4). Within the ingroup, several taxa are unresolved or resolved with weak support only. *Orthodontium lineare*, *Pyrrhobryum dozyanum*, *Goniobryum subbasilare*, *Pyrrhobryum vallis-gratiae*, and *Leptotheca gaudichaudii* do not appear in any clades with bootstrap values above 50%. *Pyrrhobryum bifarium* and *Pyrrhobryum mnioides* are resolved as sisters with weak support (bootstrap = 54%, DI = 4), while a clade containing a well-supported *Hymenodon* (bootstrap = 100%, DI = 17) is similarly weakly supported as sister to *Leptotheca boliviana* (bootstrap = 53%). In general, however, the ingroup as represented in Figure 1 is a series of well-supported clades representing either taxonomic groupings at the subfamily, family, ordinal, and superordinal levels, groups that have been recovered in previous analyses, or novel assemblages. These may be summarized as follows:

(1) Various acrocarpous clades, either well-established traditional taxa or groups that have been recovered in previous molecular phylogenetic analyses (e.g., Cox & Hedderson, 1999; Cox et al., 2000).
   (a) The Hedwigiales (bootstrap = 99%, DI = 8).
   (b) The Bryaceae/*Leptostomum* R. Br. clade (bootstrap = 79%, DI = 4).
   (c) The Orthotrichales (bootstrap = 100%, DI = 15).
   (d) The *Leptobryum* (Bruch & Schimp.)/Meesiaceae/*Tayloria* Hook. clade (bootstrap = 100%, DI = 16).
   (e) The "*Pohlia-Mnium*" clade (bootstrap = 100%, DI = 15).

   The traditional Mniaceae s.l. (Koponen, 1968) is resolved as monophyletic (bootstrap = 98%, DI = 8) and forms a larger clade (bootstrap = 100%, DI = 15) with *Pohlia* and *Mielichhoferia*. This larger grouping has previously been proposed by Cox and Hedderson (1999) and Cox et al. (2000) and appears consistently well supported in molecular analyses. The well-supported monophyly of the Mniaceae with respect to the Rhizogoniaceous exemplars contrasts with the ideas of Koponen (1988), as does the position of *Plagiomnium* sister to *Mnium hornum* and within a well-supported group including *Rhizomnium punctatum* (bootstrap = 83%, DI = 3), but excluding *Leucolepis menziesii*.

(2) The *Calomnion/Cryptopodium* clade (bootstrap = 85%, DI = 3).

   The acrocarpous bryalean genus *Calomnion* is resolved as monophyletic (bootstrap = 100%, DI = 12) and sister to the monotypic New Zealand endemic genus *Cryptopodium*, a taxon sharing many gametophytic features with *Pyrrhobryum* and, despite being acrocarpous, currently classified in the Rhizogoniaceae.

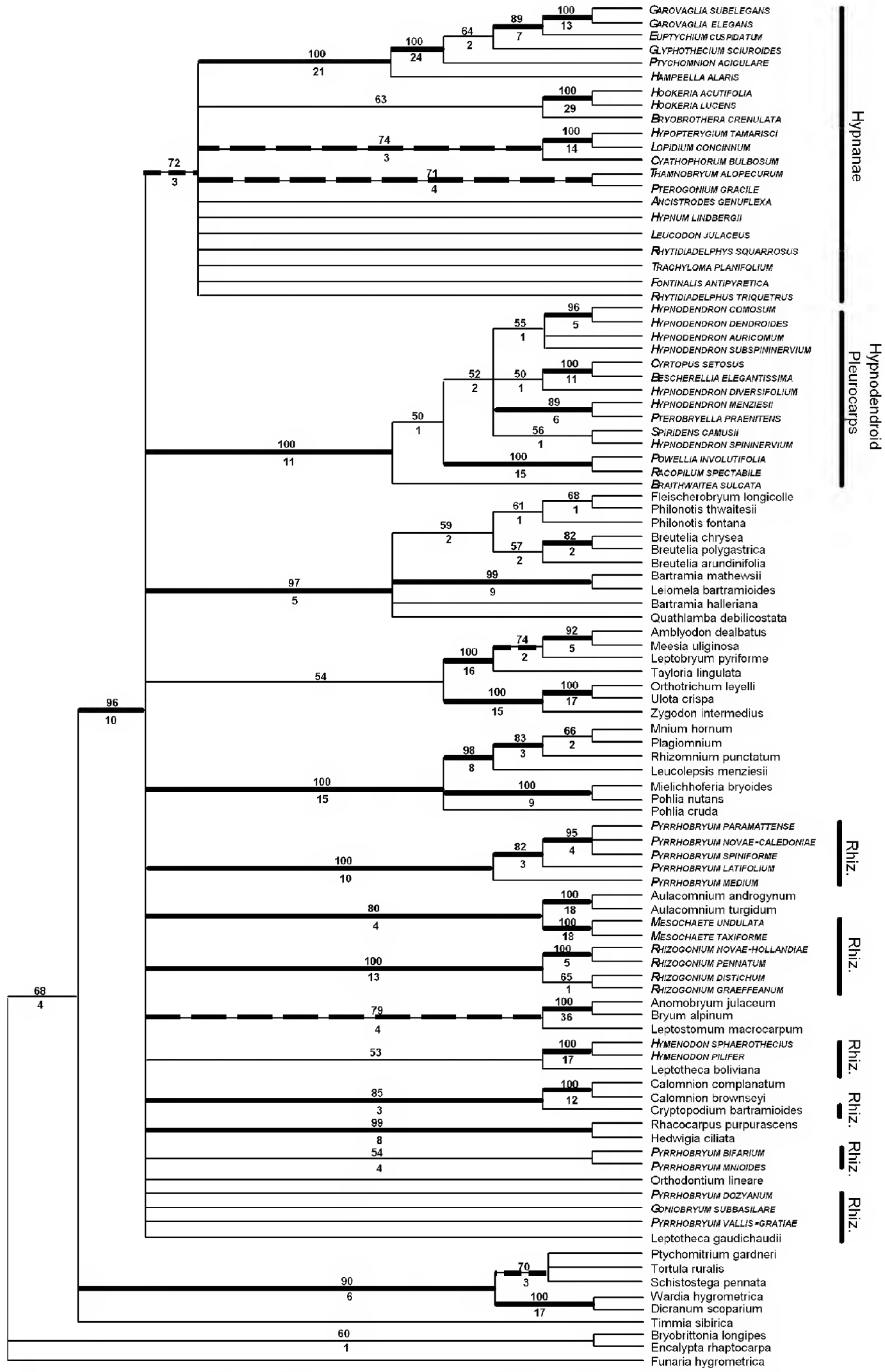

Figure 1. Bootstrap consensus tree showing supported clades (bootstrap 50% or above) based on parsimony analysis of the preliminary (*rps*4 + *rbc*L) data set. Numbers above branches are bootstrap percentages, numbers below are decay indices. Thickened branch lines indicate bootstrap values of 80% or above; dashed branch lines values of 70%–79%. Pleurocarpous taxa are in italicized capitals. Rhiz. = Rhizogoniaceae.

Table 3. Character sets used in the analysis (*rps*4 + *rbc*L, 102 taxa) to assess the phylogenetic positions of taxa missing *rbc*L sequences, showing numbers of informative characters and skewness statistic g_1 (Hillis, 1991).

Character set	Total number of characters	Informative characters	% Informative characters	g_1
Combined 2-gene matrix	1834	554	30%	−0.49
*rps*4	556	228	41%	−0.58
*rbc*L	1278	326	26%	−0.34

(3) *Rhizogonium* (bootstrap = 100%, DI = 13).

The type genus of the Rhizogoniaceae is very well supported as monophyletic.

(4) The *Aulacomnium/Mesochaete* clade (bootstrap = 80%, DI = 4).

The exemplars from *Aulacomnium* and the genus *Mesochaete* are each resolved as well-supported clades (bootstrap = 100% and DI = 18 in both cases) and as sister groups.

(5) *Pyrrhobryum* sect. *Pyrrhobryum* (bootstrap = 100%, DI = 10).

The basally ramifying members of *Pyrrhobryum* form a clade, which is itself fairly strongly resolved internally. *Pyrrhobryum medium* is sister to the other exemplars, with *P. latifolium*, in turn, sister (bootstrap = 82%, DI = 3) to a *P. paramattense* + *P. spiniforme* + *P. novae-caledoniae* clade (bootstrap = 95%, DI = 4).

(6) The Bartramiaceae (bootstrap = 97%, DI = 5).

All exemplars from the Bartramiaceae occur in a well-supported monophyletic group. This is consistent with the findings of Virtanen (2003).

(7) The hypnodendroid pleurocarps (bootstrap = 100%, DI = 11).

The recovery of this clade with very strong support is consistent with the findings of Bell and Newton (2004). As in that study, all exemplars from the Hypnodendraceae, Cyrtopodaceae, Spiridentaceae, Racopilaceae, and Pterobryellaceae together form a monophyletic group and *Hypnodendron* (Mül. Hal.) Lindb. ex Mitt. sensu Touw (1971) is paraphyletic, with *H. diversifolium* sister to the Cyrtopodaceae (although with poor support) and *H. menziesii* strongly supported as sister to *Pterobryella praenitens* (bootstrap = 89%, DI = 6). However, in this case there is little support for other relationships within the clade, other than the monophyly of the Racopilaceae, the Cyrtopodaceae, and *Hypnodendron* sect. *Comosa* (Hampe) Touw.

(8) The Hypnanae (bootstrap = 72%, DI = 3).

The exemplars from the traditional three orders of pleurocarpous mosses are resolved as a clade with only moderate support. Supported resolution within the group is generally poor, although a Ptychomniaceae/Garovagliaceae clade (also found by Newton & Cox, unpublished data) is very well supported (bootstrap = 100%, DI = 21) and strongly resolved internally. The exemplars from the Hypopterygiaceae form a clade (bootstrap = 74%, DI = 3), as do *Hookeria* J. E. Sm. and *Bryobrothera crenulata* (bootstrap = 63%, DI = 2).

THE PHYLOGENETIC POSITIONS OF *MITTENIA PLUMULA*, *PYRRHOBRYUM PUNGENS*, AND *RHIZOGONIUM LINDIGII*

With the three taxa for which *rbc*L sequences were not obtained added to the two-gene matrix, altered character statistics were as shown in Table 3. Parsimony analysis resulted in 32 MPTs of length 3213 (CI = 0.33, CI − U = 0.28, RI = 0.59). The topology of the bootstrap consensus tree (not shown) outside of the *Rhizogonium*, *Pyrrhobryum* sect. *Pyrrhobryum*, and haplolepidous clades is identical to that found after analysis of the matrix with these three taxa excluded (see Fig. 1), except that *Hypnodendron diversifolium* is not resolved as sister to the Cyrtopodaceae and is in an unresolved position in the next most inclusive clade, and *Leptotheca gaudichaudii* is similarly not resolved as sister to *Hymenodon*.

Mittenia plumula occurs in an unresolved position within the haplolepidous clade, along with *Ptychomitrium gardneri*, *Tortula ruralis*, and *Schistostega pennata*, which no longer form a group. Bootstrap support for the haplolepidous clade is reduced from 90% to 75%. *Pyrrhobryum pungens* is resolved with *P. paramattense*, *P. spiniforme*, and *P. novae-caledoniae* in a clade with a bootstrap percentage of 85 (an otherwise identical clade without *P. pungens* was recovered in the previous analysis with a bootstrap of 95%). *Rhizogonium lindigii* is resolved as sister to the *R. pennatum*/*R. novae-hollandiae* clade with a bootstrap of 55%. Bootstrap support for the grouping of *R. pennatum* and *R. novae-hollandiae* is reduced from 100% to 76%,

Table 4. Character sets used in the final study (*rps*4 + *rbc*L + *nad*5, 60 taxa) showing numbers of informative characters and skewness statistic g_1 (Hillis, 1991).

Character set	Total number of characters	Informative characters	% Informative characters	g_1
Combined 3-gene matrix	3982	725	18%	−0.68
*rps*4	575	156	27%	−0.53
*rbc*L	1283	252	20%	−0.39
*nad*5 (all)	2124	317	15%	−0.90
*nad*5 exon	1013	128	13%	−0.97
*nad*5 intron	1111	189	17%	−0.73

while support for the *R. distichum/R. graeffeanum* clade is reduced from 65% to 56%.

THREE-GENE ANALYSIS (*RPS*4 + *RBC*L + *NAD*5)

Maximum parsimony

Alignment of the three-gene data set produced a matrix of 3982 characters, of which 725 were parsimony informative. Numbers of characters, informative characters and values of g_1 for all compartments are presented in Table 4.

As with the two-gene data set, the *rps*4 region provides the greatest percentage of informative characters, although this figure (27%) is much lower than for that matrix and not greatly different from the figure for *rbc*L (20%). Hence, the larger number of taxa in the two-gene data set results in a considerably greater increase in the number of informative characters for *rps*4 than it does for *rbc*L. As the increase in taxa is mainly at the species and generic levels, this implies that *rps*4 is more informative at that phylogenetic level within the ingroup. As with the previous analysis, values of the g_1 statistic are more significant for *rps*4 than for *rbc*L.

Although the *nad*5 data provides a smaller percentage of informative characters than does the chloroplast data, the information it contains would appear to be very highly structured according to values of g_1, especially given that the absolute number of informative characters is relatively large (Hillis & Huelsenbeck, 1992). The alignment of the *nad*5 group I intron also reveals large deletions and several small insertions in *Hypnodendron comosum, H. dendroides,* and, to a lesser extent, *H. subspininervium.* These reflect substantial modification of the intron in *Hypnodendron* sect. *Comosa* in particular, corresponding to what appears to be a fundamental reorganization of secondary structure (Bell & Newton, 2004).

Analysis of the entire matrix under the parsimony criterion resulted in 24 MPTs of length 3081 (CI = 0.46, CI − U = 0.35, RI = 0.60). The strict consensus of these (including support index values) is shown in Figure 2 and a representative phylogram, illustrating relative branch lengths, in Figure 3. The consensus topology conforms to that of the two-gene analysis in that it resolves and strongly supports nodes equivalent to those that are well supported in the first analysis. In addition, a number of critical higher level nodes also receive strong to moderate support, although others remain poorly supported. Branch lengths within the pleurocarpous and proto-pleurocarpous clades are generally much shorter at the deeper nodes.

The hypnodendroid pleurocarps and the Hypnanae are both well supported (bootstrap = 100%, DI = 13 and bootstrap = 96%, DI = 8, respectively; Fig. 2). These clades are, in turn, resolved as sister groups with moderate support (bootstrap = 78%, DI = 6), thus forming a united clade of all pleurocarpous taxa excluding the Rhizogoniaceae. The topology within the hypnodendroid pleurocarp clade is consistent with that found by Bell and Newton (2004), with *Hypnodendron* emerging as strongly paraphyletic. As in that study, *Hypnodendron menziesii* is strongly supported as sister to *Pterobryella praenitens* (bootstrap = 95%, DI = 8) this group weakly supported as sister to a clade containing *Braithwaitea sulcata* and the exemplars from the Racopilaceae. The other exemplars from *Hypnodendron* form a very well-supported clade together with the Cyrtopodaceae and *Spiridens camusii* (bootstrap = 100%, DI = 31). The topology differs slightly from that of Bell and Newton (2004), in that the (*Spiridens camusii* + *Hypnodendron spininervium*) clade is sister to (*H. subspininervium* + (*H. comosum* + *H. dendroides*)) rather than to ((*Cyrtopus setosus* + *Bescherellia elegantissima*) + *H. diversifolium*). This relationship is effectively unsupported (DI = 1 only), however, as is the alternative topology in Bell and Newton (2004), which was based on considerably greater taxon sampling. Within the Hypnanae, a Ptychom-

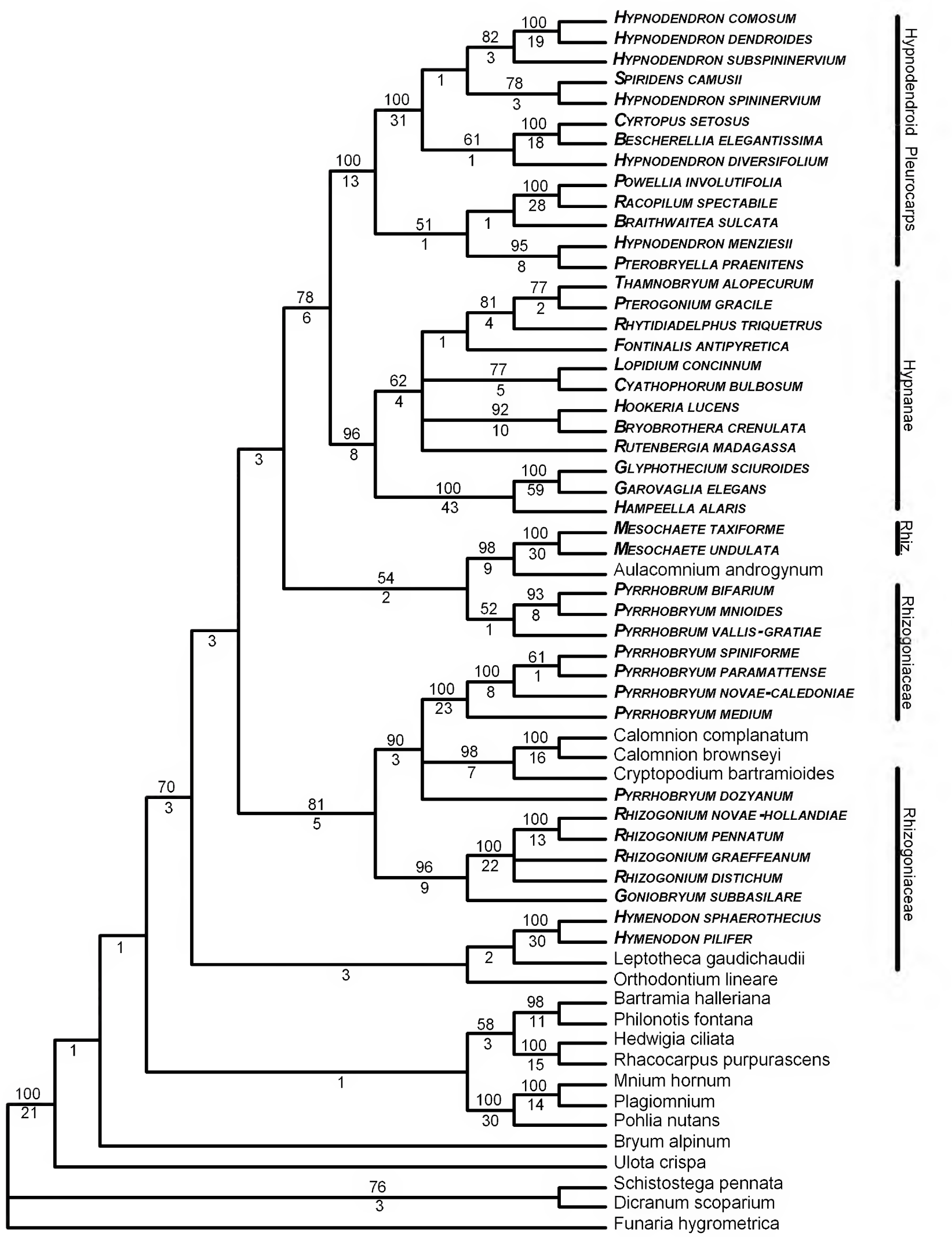

Figure 2. Strict consensus of 24 MPTs (L = 3081, CI = 0.46, RI = 0.60) based on parsimony analysis of the three-gene data set (*rps*4, *rbc*L, *nad*5). Numbers above branches are bootstrap percentages, numbers below are decay indices. Pleurocarpous taxa are in bold italicized capitals. Rhiz. = Rhizogoniaceae.

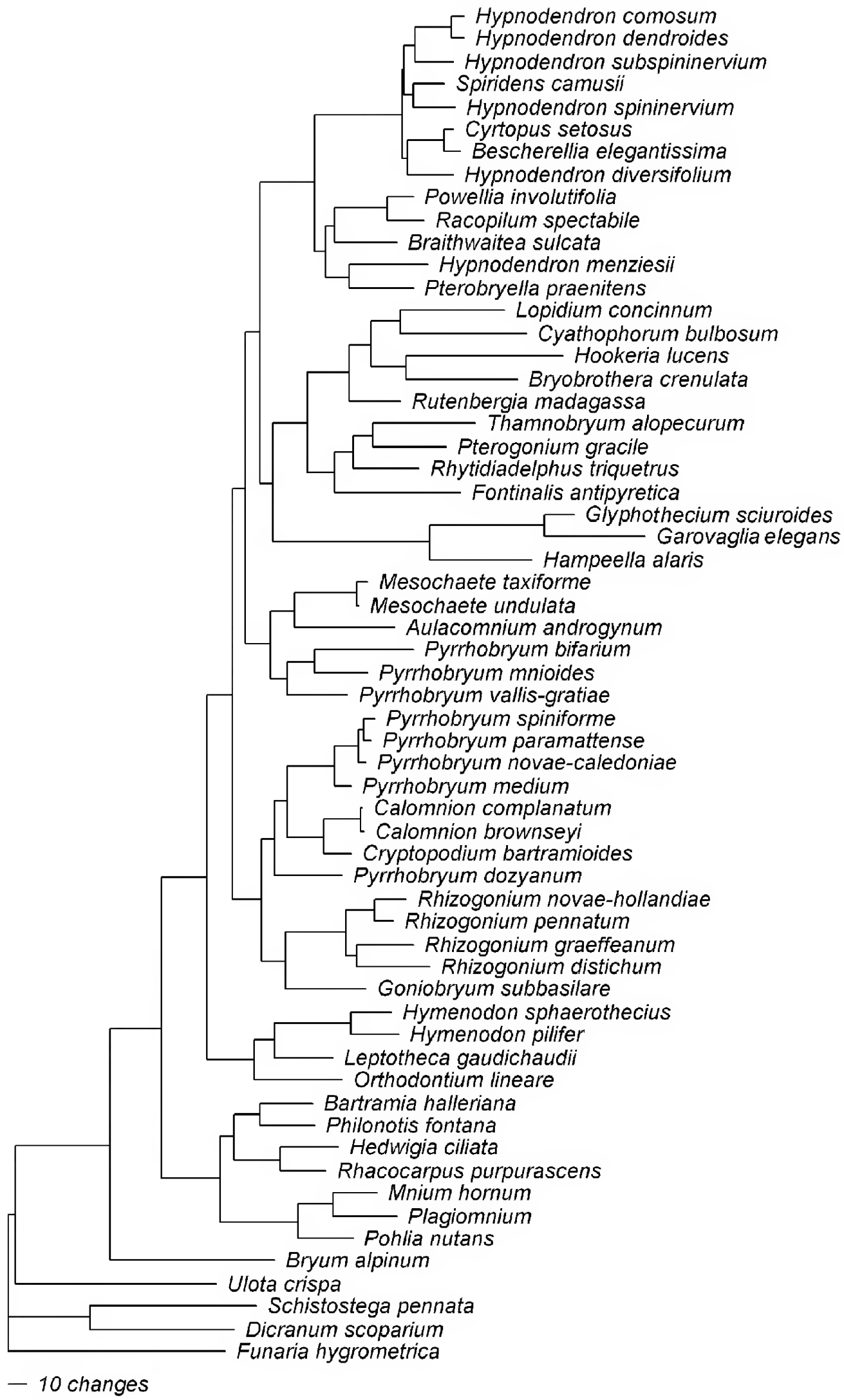

Figure 3. Phylogram of one of 24 MPTs obtained after parsimony analysis of three-gene data set (*rps*4, *rbc*L, *nad*5). Uninformative characters are included.

niaceae/Garovagliaceae clade is basal and well supported (bootstrap = 100%, DI = 43) as sister to an ambiguously resolved and rather poorly supported clade containing the remainder of the exemplars (bootstrap = 62%, DI = 4). Within the latter, *Bryobrothera crenulata* is strongly supported as sister to *Hookeria lucens* (bootstrap = 92%, DI = 10).

The Rhizogoniaceae is resolved along with the acrocarpous bryalean taxa *Calomnion*, *Aulacomnium androgynum*, and *Orthodontium lineare* as a grade basal to the Hypnanae/hypnodendroid clade. Together, these groups form a large clade separate from the other Bryalean acrocarpous taxa. The critical node supporting this group is only moderately supported, however, with a bootstrap of 70% and DI of 3 (Fig. 2). Within the rhizogoniaceaous grade there is strong support for some monophyletic groups, although the implied relationships between these are generally poorly supported.

Mesochaete is again strongly supported as sister to *Aulacomnium androgynum* (bootstrap = 98%, DI = 9; Fig. 2). This clade is, in turn, sister to a group of exemplars from *Pyrrhobryum* sect. *Bifariella*, i.e., (*Pyrrhobryum vallis-gratiae* + (*P. bifarium* + *P. mnioides*)), although this is poorly supported (bootstrap = 54%, DI = 2). The relationship of *P. bifarium* to *P. mnioides* is well supported (bootstrap = 93%, DI = 8), although the sister-group relationship of this clade to *P. vallis-gratiae* is not (bootstrap = 52%, DI = 1). This combined *Pyrrhobryum/Mesochaete/Aulacomnium* clade is resolved as sister group to the (Hypnanae + hypnodendroid pleurocarp) clade, although this is supported only by a DI value of three.

Hymenodon is monophyletic (bootstrap = 100%, DI = 30; Fig. 2) and sister to *Leptotheca gaudichaudii*, with this clade, in turn, sister to *Orthodontium lineare*. This combined *Hymenodon/Leptotheca/Orthodontium* Wilson group is sister to the rest of the Rhizogoniaceaous grade, + Hypnanae + hypnodendroid pleurocarps, although there is no other support, other than relatively low DI values, for any of these relationships.

The remainder of the Rhizogoniaceae, i.e., the bulk of the family, occurs in a large, fairly well-supported monophyletic group (bootstrap = 81%, DI = 5; Fig. 2) along with *Calomnion*. A monophyletic *Rhizogonium* (bootstrap = 100%, DI = 22) is sister to *Goniobryum subbasilare* (bootstrap = 96%, DI = 9), this clade, in turn, sister to a well-supported *Pyrrhobryum/Cryptopodium/Calomnion* clade (bootstrap = 90%, DI = 3). All of the exemplars from *Pyrrhobryum* sect. *Pyrrhobryum* form a securely supported monophyletic group (bootstrap = 100%, DI = 23), while *Calomnion* is well supported as sister to *Cryptopodium bartramioides* (bootstrap = 98%, DI = 7). Interestingly, *Pyrrhobryum dozyanum*, a member of *Pyrrhobryum* sect. *Bifariella*, is securely placed within the larger *Pyrrhobryum* sect. *Pyrrhobryum/Cryptopodium/Calomnion* clade (in an unresolved position), and does not appear as closely related to the other taxa in section *Bifariella* (see above). There is well-supported resolution within both *Rhizogonium* and *Pyrrhobryum* sect. *Pyrrhobryum*. Within *Rhizogonium*, *R. pennatum* and *R. novae-hollandiae* are sisters (bootstrap = 100%, DI = 13), while the positions of *R. graeffeanum* and *R. distichum* are unresolved. In *Pyrrhobryum* sect. *Pyrrhobryum*, *P. medium* is sister to the other taxa, with *P. novae-caledoniae* sister (bootstrap = 100%, DI = 8) to *P. spiniforme* + *P. paramattense* (bootstrap = 61%, DI = 1).

Maximum likelihood

In the analysis of the three-gene data set under the likelihood criterion the GTR + G + I model of evolution was assumed, the most complex suggested by Modeltest for any of the individual compartments and the "best fit" model for the combined data set. The analysis resulted in a tree with a log-likelihood score of 22,467.78978 (Fig. 4).

The general topology of the ML tree corresponds to that of the MP trees, although there are a number of differences within individual clades. High-level relationships within the ingroup are identical, i.e., corresponding to (((Hypnanae + hypnodendroid pleurocarps) + (*Mesochaete/Aulacomnium/Pyrrhobryum* sect. *Bifariella*)) + (*Goniobryum/Rhizogonium/Cryptopodium/Calomnion/P.* sect. *Pyrrhobryum/P. dozyanum*)) + (*Hymenodon/Leptotheca/Orthodontium*). Some of these nodes are poorly supported in the MP analysis, and thus corroboration under the likelihood criterion could potentially be perceived as increasing confidence in the topology.

Within the Hypnodendroid pleurocarp group, the *Spiridens camusii* + *Hypnodendron spininervium* clade is sister to the Cyrtopodaceae. This is at odds with the results of the MP analysis, but consistent with the topology found by Bell and Newton (2004), excepting the unresolved position of *Hypnodendron diversifolium*, which is sister to the Cyrtopodaceae in the latter. Within the Hypnanae, *Rutenbergia madagassa* is sister to a *Thamnobryum alopecurum* + *Pterogonium gracile* + *Rhytidiadelphus triquetrus* + *Fontinalis antipyretica* clade, this larger clade, in turn, sister to the hookeriaceous exem-

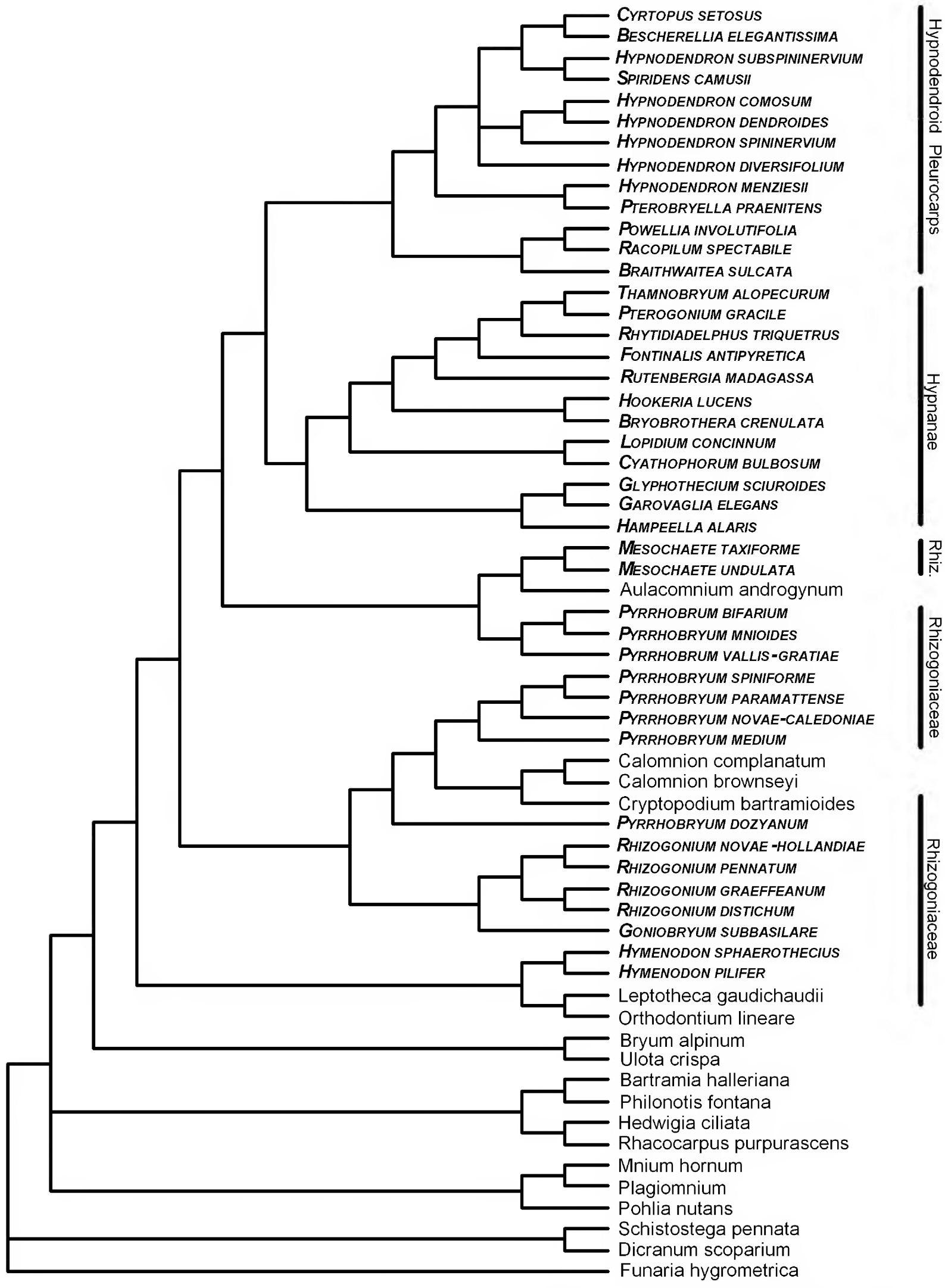

Figure 4. Tree found after analysis of the three-gene data set (*rps*4, *rbc*L, *nad*5) under the likelihood criterion (−ln = 22,467.78978). Pleurocarpous taxa are in italicized capitals. Rhiz. = Rhizogoniaceae.

plars, with the Hypopterygiaceae sister to all of these taxa together. Although taxon sampling within the Hypnanae is too low for internal topology to be particularly meaningful, it may be noted that the pattern is not inconsistent with the MP analysis, being recovered in four out of the 24 MPTs (not shown). Similarly, the increased resolution of the ML tree within the large rhizogoniaceous clade compared with Figure 2 also corresponds to some of the topologies found under the parsimony criterion. In 12 of the MPTs *Pyrrhobryum dozyanum* is sister to (*Pyrrhobryum* sect. *Pyrrhobryum/Calomnion/Cryptopodium*) as in the ML tree, while in 12 also *Rhizogonium graeffeanum* is sister to *R. distichum*. In six MPTs (not shown) both of these topologies occur together.

Other differences between the ML and MP topologies include the positions of *Orthodontium lineare* and *Leptotheca gaudichaudii*, which are sisters in the ML tree, while *Orthodontium* is basal to a *Leptotheca* + *Hymenodon* clade in the MP trees. Outside of the Rhizogoniaceous grade, the "*Pohlia–Mnium*" clade is not resolved as sister to the combined Bartramiaceae/Hedwigiales group in the ML tree as it is in the MP trees, and the sister group to the (Rhizogoniaceous grade + Hypnanae + hypnodendroid pleurocarps) is a *Bryum alpinum* + *Ulota crispa* clade in the ML tree, as contrasted with the Mniaceous/Bartramiaceous clade in the MP trees.

Comparison of chloroplast and mitochondrial data sets

The partition homogeneity test resulted in a *P* value of 0.02 for the chloroplast and mitochondrial data, which, theoretically, is sufficient to reject the hypothesis that the two data sets are congruent at the 5% probability level, if not at the 2% level. This may be considered an ambiguous result, given that interpretation of this test is controversial. A highly significant result would simply indicate that combining the data from the two partitions would have a significant effect on overall levels of homoplasy (Yoder et al., 2001). This need not necessarily equate to a decrease in phylogenetic accuracy, however. Congruence between two genomic compartments is better assessed by examining the extent to which independently derived topologies conflict with each other in a manner that is well supported (Eldenäs & Linder, 2000; Goldblatt et al., 2002).

The separate analysis of the chloroplast data set under the parsimony criterion resulted in eight trees of length 2017 (CI = 0.41, CI − U = 0.32, RI = 0.54), while analysis of the mitochondrial data set produced four trees of length 1035 (CI = 0.59, CI − U = 0.45, RI = 0.72). Strict consensus trees for both analyses are shown in Figure 5 and representative phylograms in Figure 6.

There are a number of topological differences between the chloroplast and mitochondrial trees, although these are without exception weakly supported (see discussion). The strict consensus of the chloroplast trees is largely unresolved with regard to relationships between the major pleurocarpous clades, which is consistent with the lack of supported resolution in this area in the two-gene analysis. As in the combined analysis, the *Mesochaete/Aulacomnium* clade is resolved as sister to a clade consisting of three members of *Pyrrhobryum* sect. *Bifariella* (*P. bifarium*, *P. mnioides*, and *P. vallis-gratiae*), although supported by a DI value of 2 only (see Fig. 5A). This clade is, in turn, resolved as sister to a group consisting of the hypnodendroid pleurocarps and *Hymenodon*, although both this relationship and the position of *Hymenodon* are only supported by a DI of 1. The mitochondrial tree, by contrast, resolves the hypnodendroid pleurocarps and the Hypnanae as sister groups (in common with the combined analysis), with moderate support (bootstrap = 70%, DI = 3; Fig. 5B), and fails to recover the *Mesochaete/Aulacomnium/Pyrrhobryum*/section *Bifariella* clade. Both data sets resolve *Goniobryum* as sister to a monophyletic *Rhizogonium*, but only the mitochondrial trees place this clade as sister to the *Pyrrhobryum* sect. *Pyrrhobryum/Calomnion/Cryptopodium/Pyrrhobryum dozyanum* group (with a bootstrap of 65% and DI of 1; Fig. 5B). *Pyrrhobryum dozyanum*, which is in an unresolved position within this clade in the combined analysis, is sister to the entire group in the chloroplast trees (bootstrap = 54%, DI = 19) and sister to *Pyrrhobryum* sect. *Pyrrhobryum* in the mitochondrial trees (bootstrap = 76%, DI = 2). Topography within *P.* sect. *Pyrrhobryum* is consistent between the two data sets, but better resolved and supported in the mitochondrial trees.

Both sets of trees in Figure 5 resolve a large clade of pleurocarps and bryalean acrocarps that excludes the *Pohlia*/Mniaceae group, Bartramiaceae, and Hedwigiales, although in the case of the chloroplast trees this includes *Bryum alpinum* and *Ulota crispa* forming a sister group to the Hypnanae (effectively with no support). The Hypnanae itself is considerably better supported in the mitochondrial trees (bootstrap = 81%, DI = 3; Fig. 5B) than in the chloroplast trees (bootstrap = 58%, DI = 3). Within the Hypnanae, the mitochondrial data resolves the Ptychomniaceae/Garovagliaceae clade

as sister to the remainder of the Hypnanae (as in the combined analysis), and supports the monophyly of the latter with a bootstrap of 69% and a DI value of 2. In the chloroplast trees, the Ptychomniaceae/Garovagliaceae clade is sister to the Hypopterygiaceae, although supported only by a DI of 1. Other topological differences between the two sets of trees in the Hypnanae are similarly unsupported. Topological differences within the hypnodendroid pleurocarps generally correspond to scenarios which are discussed in a separate study (Bell & Newton, 2004). The exceptionally long branch supporting the monophyly of the hypnodendroid pleurocarps in the *nad*5 phylogram (Fig. 6B) corresponds to rapid evolution of both the *nad*5 exon and intron within this group (Bell & Newton, 2004).

Discussion

The results allow a hypothesis of phylogenetic relationships amongst the major groups of pleurocarpous and closely related acrocarpous mosses to be constructed. A phylogenetic hypothesis need not necessarily correspond exactly to an individual MPT or consensus topology, but rather should (1) represent an individual systematist's best estimate of phylogeny given all of the available evidence, and (2) be sufficiently explicit to be testable.

In the case of this study it would not be appropriate to present a single MPT as a hypothesis of relationships for the following reasons:

(1) All trees obtained contain a number of nodes that are poorly supported.
(2) Not all of the inferences that can be made by considering all of the available evidence are represented on any single cladogram.

The phylogenetic hypothesis is presented in Figure 7. This discussion will focus primarily on the basis for this hypothesis and the more immediate aspects of its interpretation within the context of traditional concepts of relationships and morphology. Detailed discussion of morphological characters and scenarios for character evolution, particularly within the Rhizogoniaceae, will be the subject of future studies.

The vast majority of the pleurocarpous mosses occur in a large apical clade, which, in turn, comprises two sister groups, the Hypnanae sensu Buck and Goffinet (2000) and the hypnodendroid pleurocarps sensu Bell (Bell & Newton, 2004). These clades are heavily asymmetrical in terms of numbers of extant species, the Hypnanae containing approximately 6400 species (based on Walter, 1983) in about 460 genera and 52 families (Buck & Goffinet, 2000), the hypnodendroid pleurocarps 60 to 70 species, currently in 9(10) genera and 5(6) families (but see Bell & Newton, 2004). The Hypnanae is defined by a combination of characters including the production of perichaetia on highly reduced secondary modules (Newton & De Luna, 1999) that are produced laterally from primary modules non-specifically (i.e., neither obviously basally nor apically) and a reduced costa that is entirely or nearly entirely homogenous in transverse section (Hedenäs, 1994). Nearly all of the Hypnanae have a creeping growth form or else are tufted, pendent, or dendroid from a partially creeping primary stem. A large proportion also exhibit non-determinate growth of the primary module (Newton & De Luna, 1999). The hypnodendroid pleurocarps share with the Hypnanae (and some Rhizogoniaceae) the production of perichaetia on highly reduced secondary modules non-specifically, and are further characterized by having a strong costa that is clearly differentiated into stereids and deuters and, except in Racopilaceae, tufted growth of the primary module, which reiterates from the extreme base without creeping. The primary module is more or less vegetatively determinate, growth being effectively constrained by the overall architecture of the plant, which is commonly dendroid. The dendroid form is probably plesiomorphic within the group (see Bell & Newton, 2004). Plants are often very robust and occur terrestrially or as epiphytes in southern temperate or montane tropical rainforest.

The sister-group relationship of the Hypnanae to the hypnodendroid pleurocarps is moderately well supported in the three-gene analysis. Although it is not resolved by the chloroplast data separately, it occurs in the mitochondrial MPTs and support values are increased in the combined analysis, suggesting that there may be compatible signal in the chloroplast data that is congruent with the mitochondrial topology.

The sister-group relationship of *Mesochaete* and *Aulacomnium* is consistently well supported and is in agreement with the findings of O'Brien (in review). O'Brien sampled extensively from *Aulacomnium* and resolved *A. heterostichum* as sister to the remainder of the genus. This is significant, as the grouping of *Mesochaete* and *Aulacomnium*, which initially seems incongruous, appears less so if the plesiomorphic traits within *Aulacomnium* are assumed to be those of *A. heterostichum* (O'Brien, in review, pers. comm.). *Aulacomnium heterostichum* has a similar leaf morphology to *Mesochaete* and

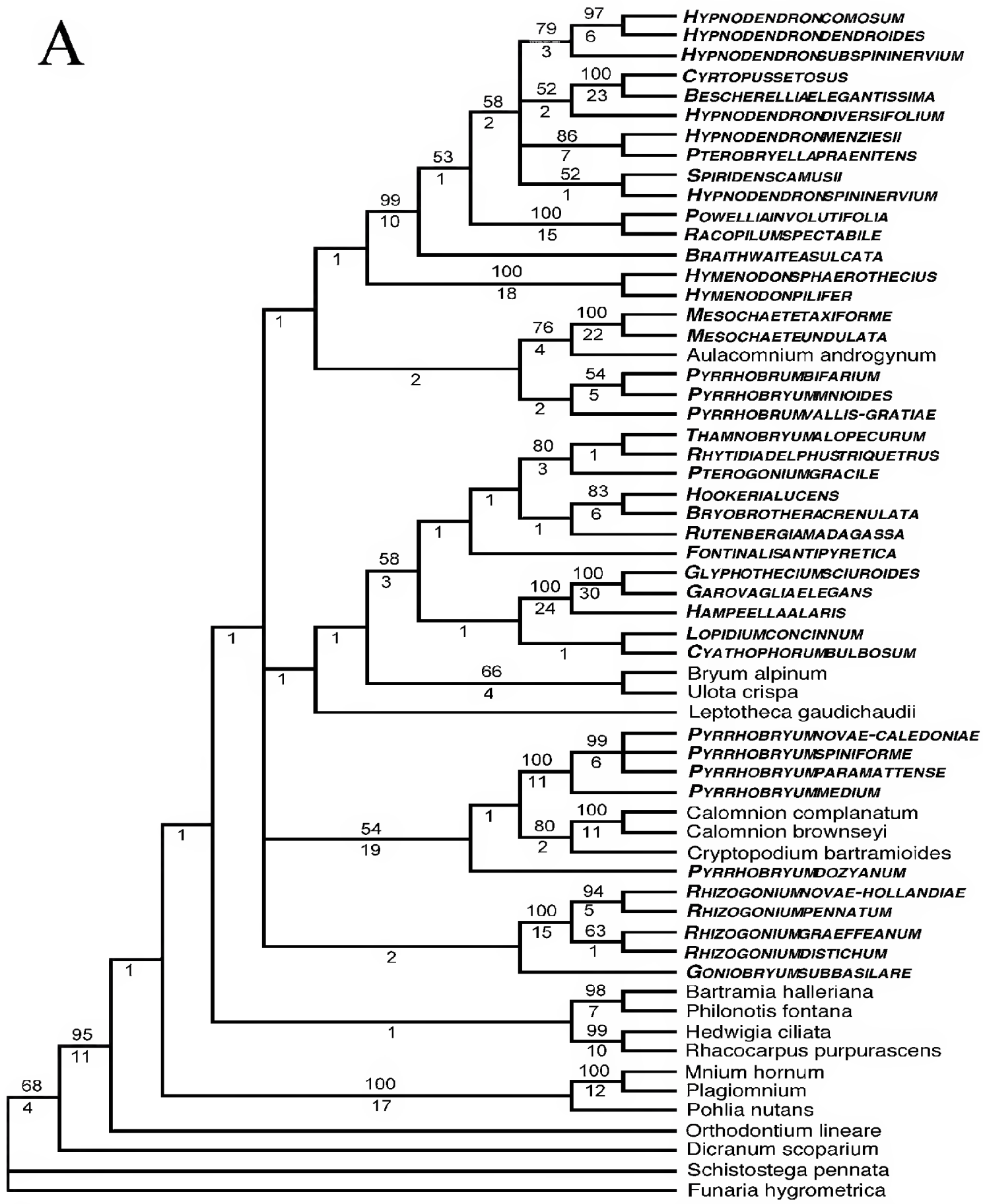

Figure 5. —A. Strict consensus of eight MPTs (L = 2017, CI = 0.41, CI(U) = 0.32, RI = 0.54) obtained from analysis of the chloroplast data set (*rps*4 + *rbc*L). —B. Strict consensus of four MPTs (L = 1035, CI = 0.59, CI(U) = 0.45, RI = 0.72) obtained from analysis of the mitochondrial data set (*nad*5). Numbers above branches are bootstrap percentages, numbers below are decay indices. Pleurocarpous taxa are in italicized capitals.

occurs on similar soil types, while both taxa have deciduous apical leaves and sulcate capsules (O'Brien, in review, pers. comm.).

A clade containing all but one of the exemplars from *Pyrrhobryum* sect. *Bifariella* as sister to the *Aulacomnium/Mesochaete* group is consistently resolved by the chloroplast data (and appears in the trees from the combined analysis), but is poorly supported. *Pyrrhobryum bifarium* is well supported as sister to *P. mnioides*, although *P. vallis-gratiae* is only tentatively supported as sister to that clade. *Pyrrhobryum vallis-gratiae* is endemic to South Africa and is one of only two species of *Pyrrhobryum* (O'Shea, 1997) and three Rhizogoniaceae occurring in Africa. In the mitochondrial trees, the *Aulacomnium/Mesochaete* group, *Pyrrhobryum vallis-gra-*

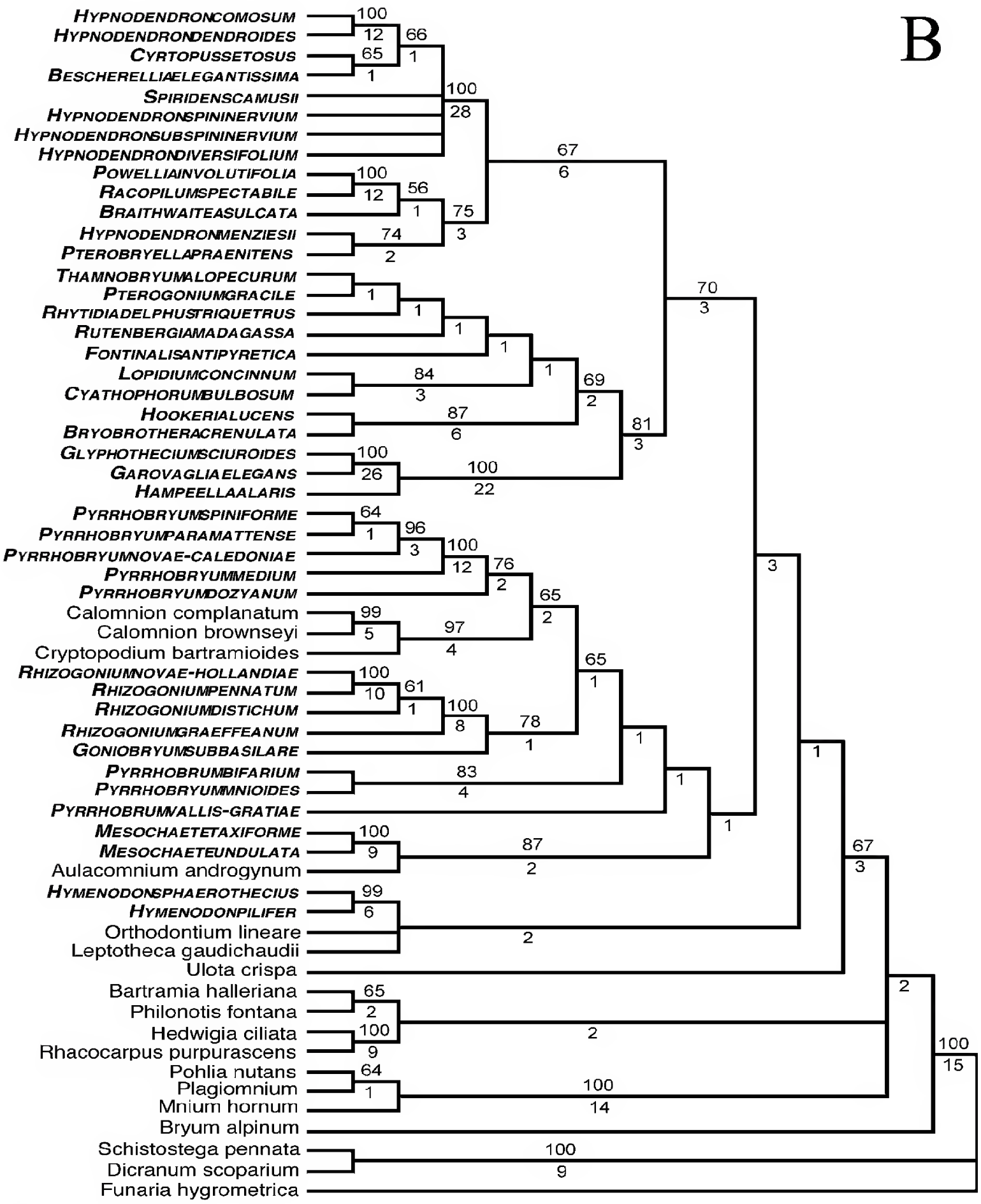

Figure 5. Continued.

tiae, and *P. mnioides/P. bifarium* form a grade basal to the largest rhizogoniaceous clade, although all of the relevant nodes are supported only by DI values of 1.

The *Mesochaete/Aulacomnium/Pyrrhobryum* sect. *Bifariella* clade, when it is resolved, is often sister to the (Hypnanae + hypnodendroid pleurocarps), as in Figure 2. In the chloroplast trees as summarized in Figure 5 it is basal (with *Hymenodon*) to the hypnodendroid pleurocarps, although this is unsupported (DI = 1) and at odds with the well-supported (Hypnanae + hypnodendroid pleurocarps) clade in the combined analysis (Fig. 2). In other MPTs obtained from incomplete versions of the chloroplast data set during pilot studies, the *Mesochaete/Aulacomnium/Pyrrhobryum* sect. *Bifariella* clade was often sister to the (Hypnanae + hypnodendroid pleurocarps), as in the three-gene

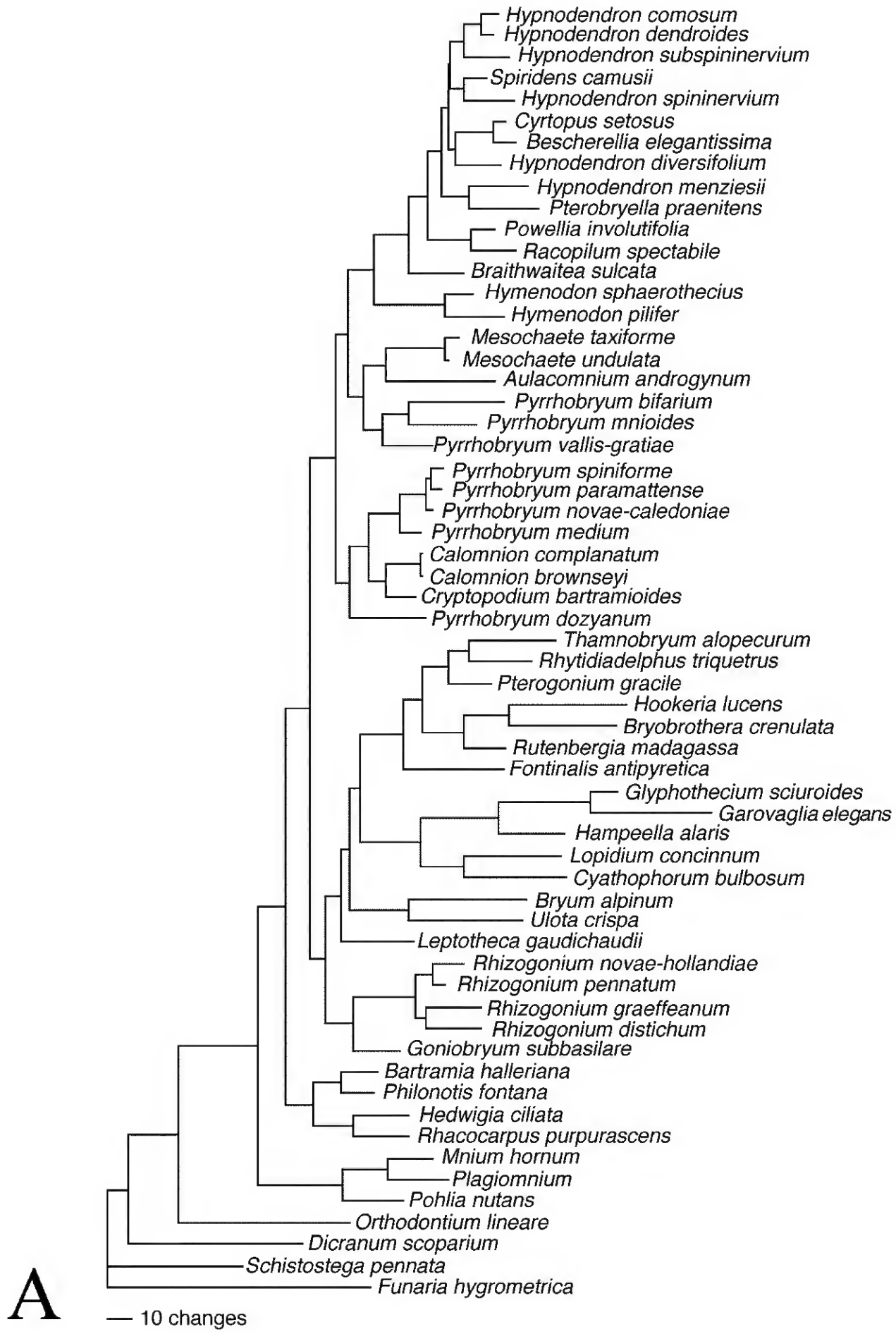

Figure 6. —A. Phylogram of one of eight MPTs obtained from analysis of the chloroplast data set (*rps*4 + *rbc*L). —B. Phylogram of one of four MPTs obtained after analysis of the mitochondrial data set (*nad*5). Uninformative characters are included.

analysis. This hypothesis is discussed further below.

One of the most significant well-supported hypotheses deriving from this study is the monophyly of a large clade of rhizogoniaceous exemplars, including *Calomnion*. The clade includes all of the Rhizogoniaceae that show the unique form of pleurocarpy characterized by the production of reduced perichaetial modules basally, i.e., *Rhizogonium* and *Pyrrhobryum* sect. *Pyrrhobryum* (both of which are monophyletic). *Cryptopodium* and *Calomnion* form a small acrocarpous clade that otherwise seems rather incongruous. *Cryptopodium* shares many gametophytic features with *Pyrrhobryum*, differing

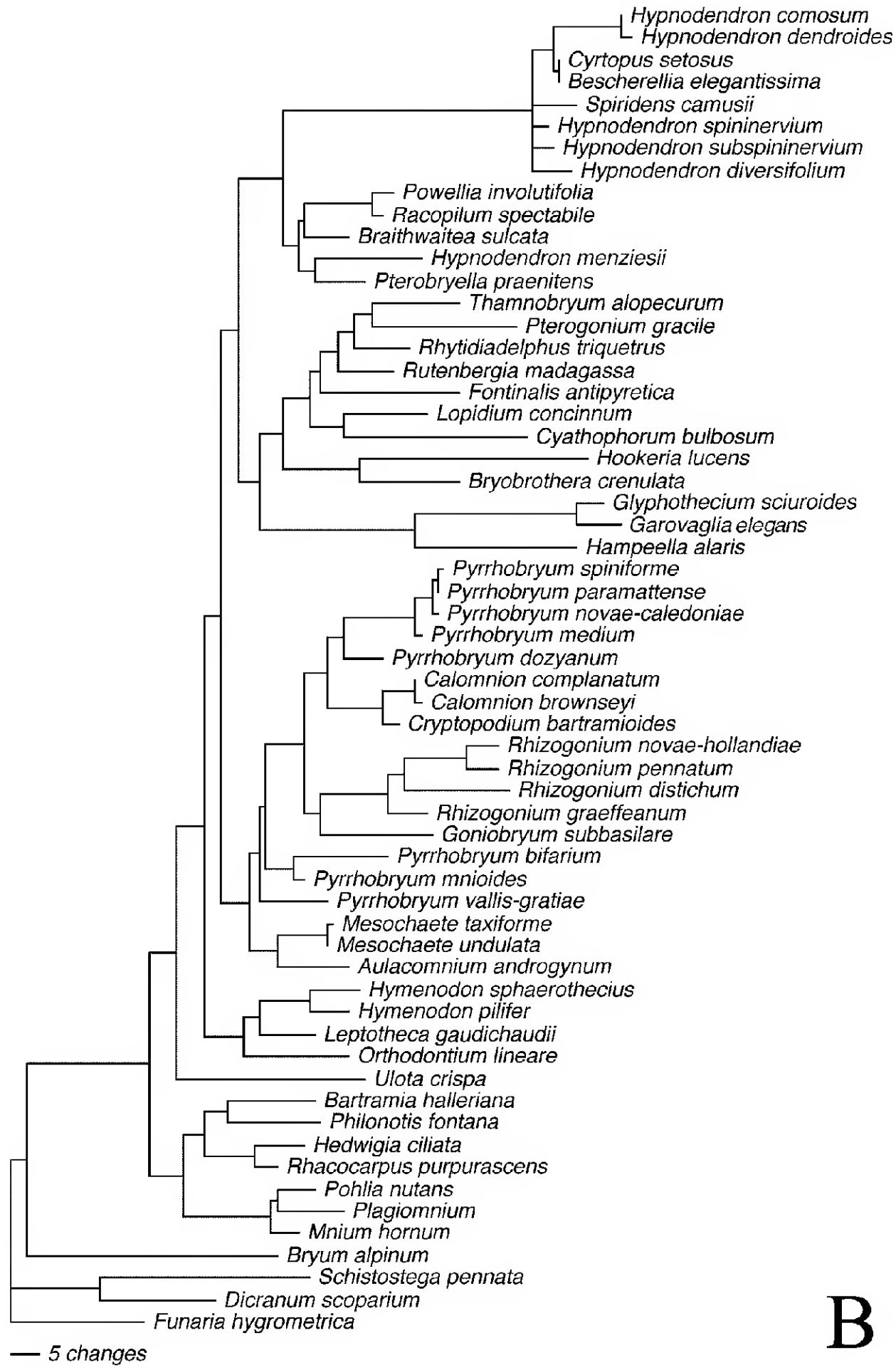

Figure 6. Continued.

primarily in being acrocarpous and more robust, while *Calomnion* is a diminutive genus of strongly complanate, heterophyllous plants (see Vitt, 1995). A very interesting result is the placement of *Pyrrhobryum dozyanum* in the larger clade, close to *Pyrrhobryum* sect. *Pyrrhobryum*. *Pyrrhobryum dozyanum*, a member of *P.* sect. *Bifariella*, has reduced perichaetial modules produced non-specifically, and would be expected to be closely related to *P. bifarium*, *P. mnioides*, and *P. vallis-gratiae*. However, a monophyletic *Pyrrhobryum* sect. *Bifariella* is 20 steps longer than the most parsimonious topologies summarized in Figure 2. *Pyrrhobrum dozyanum* is in an unresolved position in relation to *P.* sect. *Pyrrhobryum* and the *Calomnion/Cryptopodium* clade in the strict consensus of trees de-

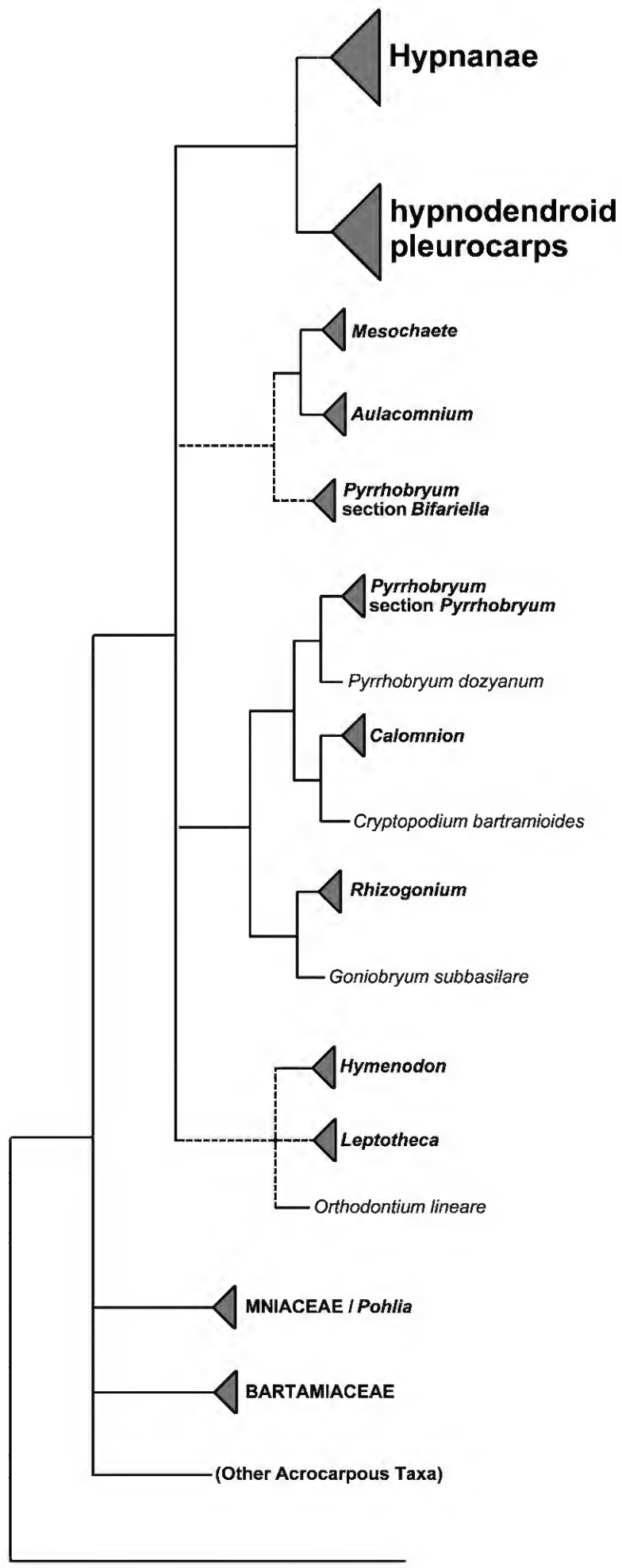

Figure 7. Hypothesis of higher level phylogenetic relationships amongst the major clades of pleurocarpous mosses. Relationships regarded as well supported are indicated by solid lines, those which are tentatively proposed by dashed lines.

rived from the three-gene analysis, while the chloroplast trees place it as sister to (*Pyrrhobryum* sect. *Pyrrhobryum* + (*Calomnion* + *Cryptopodium*)) and the mitochondrial data resolves it as sister to *P.* sect. *Pyrrhobryum*. These two alternative topologies are each found in exactly half of the MPTs from the three-gene analysis. However, while the chloroplast topology is unsupported (DI = 1), the mitochondrial topology has moderate support (bootstrap = 76, DI = 2). The latter also seems slightly more congruent with the distribution of morphological characters, and thus we hypothesize that this currently represents the best estimate of the phylogenetic position of *P. dozyanum*, as indicated in Figure 7. This is, however, at odds with the results of the ML analysis, in which *P. dozyanum* is sister to (*P.* sect. *Pyrrhobryum* + (*Calomnion* + *Cryptopodium*)). As the large clade of (*Pyrrhobryum* sect. *Pyrrhobryum/P. dozyanum/Calomnion/Cryptopodium/Rhizogonium/Goniobryum*) is well supported as monophyletic, we propose the informal name "core rhizogonioids" to refer to the group.

Turning briefly to relationships within the Hypnanae, it is clear that there is a lack of resolution in the MP consensus tree compared with the ML topology. This may be partially a consequence of disproportionately long branches (in turn, probably a result of inadequate taxon sampling within this very large group, see Fig. 3), leading to conflict between phylogenetic signal and artifactual signal produced by the phenomenon of long branch attraction (Felsenstein, 1978). Phylogeny within this group, however, is outside the scope of this study.

The paraphyly of *Hypnodendron* and the apparent derivation of the Spiridentaceae and Cyrtopodaceae from within a clade of *Hypnodendron* species is a dramatic hypothesis, discussed at length in Bell and Newton (2004). Optimization of architectural characters onto a phylogenetic hypothesis derived from more detailed taxon sampling in that study suggests that the simple, unbranched forms of the Spiridentaceae and Cyrtopodaceae are apomorphic, probably representing independent adaptive shifts towards epiphytism. The Racopilaceae are also derived from within the hypnodendroid pleurocarps and represent an abandonment of the tufted hypnodendroid architecture in favor of a prostrate, creeping form very reminiscent of many of the Hypnanae.

A clade comprising *Hymenodon*, *Leptotheca*, and *Orthodontium lineare* appears in the trees from the three-gene analysis and in the mitochondrial consensus, although these taxa are widely scattered in the chloroplast trees (note, however, that despite their disparate positions they are not separated by any nodes supported by the bootstrap or by DI val-

Table 5. Effects of constraining the monophyly of the (Hypnanae + hypnodendroid pleurocarps) with selected other groups using the three-gene dataset (*rps*4 + *rbc*L + *nad*5, 60 taxa).

Group or taxon constrained as sister to Hypnanae + hypnodendroid pleurocarps	Deviation from most parsimonious topologies
(*Pyrrhobryum* section *Pyrrhobrum* + *P. dozyanum* + *Calomnion* + *Cryptopodium* + *Rhizogonium* + *Goniobryum*)	+ 5 steps
(*Aulacomnium* + *Mesochaete*)	+ 3 steps
(*Pyrrhobryum bifarium* + *P. mnioides* + *P. vallis-gratiae*)	+ 2 steps
Pyrrhobryum vallis-gratiae	+ 5 steps
(*Pyrrhobryum bifarium* + *P. mnioides*)	+ 3 steps
(*Hymenodon* + *Leptotheca* + *Orthodontium*)	+ 4 steps
Leptotheca gaudichaudii	+ 7 steps
Orthodontium lineare	+ 9 steps
Hymenodon	+ 5 steps

ues above 1). This clade is thus tentatively proposed as a phylogenetic hypothesis, although it remains far from secure. It is notable that in the two-gene analysis, in which two species of *Leptotheca* were included, the genus is not supported as monophyletic (as in the likelihood topology of Cox & Hedderson, 1999), although no supported nodes are inconsistent with its monophyly.

It is regrettable that in the strict consensus of the three-gene data set (Fig. 2) there is no strongly supported basis for confidently proposing a sister group to the (Hypnanae + hypnodendroid pleurocarp) clade. This critically important question was investigated further by forcing a number of candidates for sisterhood into monophyly with the (Hypnanae + hypnodendroid pleurocarps) in the three-gene analysis and observing the effects on tree length (see Table 5). On this basis, if the (*Mesochaete* + *Aulacomnium* + *Pyrrhobryum* sect. *Bifariella*) clade is rejected as being most closely related to the (Hypnanae + hypnodendroid pleurocarps), the next most parsimonious sister group is *P.* sect. *Bifariella* s. str., i.e., (*P. bifarium* + *P. mnioides* + *P. vallis-gratiae*). This is an interesting observation, as there are considerable structural similarities between these taxa *(P. bifarium* in particular) and many of the hypnodendroid pleurocarps. *Pyrrhobryum bifarium* is in structural terms a miniature *Hypnodendron* with a *Pyrrhobryum* leaf morphology, in that it has immediately orthotropous primary modules that reiterate from the base, vegetative secondary modules produced from the upper part of the primary module (giving a dendroid morphology), a primary module in which the leaf morphology often changes to mimic that of the secondary modules after these are produced, and highly reduced secondary perichaetial modules. In conclusion, therefore, it would seem likely that the hypothesized (*Mesochaete* + *Aulacomnium* + *Pyrrhobryum* sect. *Bifariella*) clade, or else a subset of the taxa it contains, is the sister group to the large apical pleurocarpous clade.

The lack of well-supported resolution in the "backbone" of the rhizogoniaceous grade implies that the initial diversification of the basal pleurocarpous groups may have occurred relatively rapidly. In both the mitochondrial and the chloroplast phylograms (Fig. 6), branches are very short in this zone, both relative to those supporting apical clades and those at deeper nodes. It is apparent that the combined data set does contain a wide range of phylogenetic signal, as the chloroplast and mitochondrial characters provide support at different levels (Fig. 5) and well-supported resolution is found at both ancient and recent dichotomies. In this context, an inability to resolve nodes at intermediate levels suggests that the rate of cladogenesis and the rate of molecular evolution may be uncoupled at this point. Mishler (1994) and Mishler et al. (1988) describe the concept of a window of informativeness for a set of data. While a slowly evolving character set does not produce sufficient signal to allow phylogeny to be resolved, a region that is evolving extremely fast may cause problems (i.e., with saturation) due to multiple changes in the same character (Mishler, 1994). If a radiation has occurred very rapidly and is also fairly ancient, it is possible that no character set that evolves relatively constantly throughout time will provide a window capable of illuminating it. This can be interpreted as an argument for using morphological data to help resolve ancient polytomies in molecular phylogenies displaying the type of pattern described above. The very nature of morphological

change, almost by definition, is that it speeds up during periods of lineage diversification and slows down at other times (Bateman, 1999). Hence, assuming that problems of interpretation can be overcome, the phenotype may retain imprints of ancient phylogenetic events that phylogenetically tractable areas of genotype have either "missed" or "forgotten." It is thus possible that key morphological characters may eventually provide the solution to resolution between the major clades within the rhizogoniaceous grade.

Although taxonomic description was not an objective of this study, fundamental taxonomic changes will be required at all levels in the "basal" pleurocarpous mosses as a consequence of the patterns of relationships revealed. Even within the context of the limited phylogenetic information available at the time, the classification of Buck and Goffinet (2000) recognized several paraphyletic groups as higher level taxa. Given the pectinate nature of the phylogeny of the diplolepidous-alternate mosses in relation to definitive innovations, this is inevitable if any continuity with traditional classifications is to be maintained. Given that changes will be controversial, however, it is preferable that all aspects of phylogeny in the non-hypnanaean pleurocarpous groups should be securely resolved before they are implemented.

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A SIMPLE TAXONOMIC TREATMENT FOR A COMPLICATED EVOLUTIONARY STORY: THE GENUS *HYGROAMBLYSTEGIUM* (HYPNALES, AMBLYSTEGIACEAE)[1]

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Abstract

Taxonomic issues associated with plant plasticity and convergence are especially acute in aquatic environments. The potential of comparative methods for testing the evolutionary significance of morphological characters that have traditionally been used for species delineation is presented in the context of the increasing availability of molecular phylogenies. In the aquatic moss genus *Hygroamblystegium,* most of the morphological variation results from plasticity. The vast majority of the characters that have traditionally been used for taxonomy are highly intercorrelated and exhibit accelerated evolution rates with little phylogenetic association. It is, therefore, suggested that the *Hygroamblystegium* species as formerly circumscribed can be abandoned and should be synomized with *H. varium.*

Key words: *Amblystegium,* comparative methods, convergence, *Hygroamblystegium,* molecular phylogenetics, plasticity, synomization.

Convergence, as the similarity between organisms that is not the direct result of shared phylogenetic history (and that may instead result from independent adaptations to similar environments), is a fundamental issue in systematic biology (Evans et al., 2003; Jousselin et al., 2003; Wiens et al., 2003). With the increasing development of molecular phylogenies, methodological approaches that allow for testing the mode, tempo, and phylogenetic association of morphological evolution have been statistically formalized so that it is now possible to infer historical patterns of evolution from the phylogeny and morphology from the current taxa (Martins & Hansen, 1997; Pagel, 1997, 1999; Schluter et al., 1997). In many cases, reconstructing morphological evolution from molecular phylogenies revealed complicated patterns, raising issues regarding the appropriate taxonomic treatment for clades that are solely, or almost solely, defined based on molecular features (see, e.g., Swann et al., 1999).

These issues are especially acute when, in addition to convergence, morphological variation is also prone to plasticity (e.g., Ghebrehiwet, 2000; Hansen et al., 2000), a phenomenon that is very common in aquatic environments (Kaplan, 2002). Most of the taxonomy of aquatic angiosperms is, therefore, based on flower and fruit morphology, which are assumed to be less plastic and convergent than the traits of the vegetative system that is permanently shaped by growth in a constraining environment. Similarly, in mosses, the bulk of the taxonomy has traditionally been based on sporophytic features. In many aquatic mosses, however, sporophyte morphology is shared among species of the main genera (e.g., *Fontinalis* Hedw., *Hygroamblystegium* Loeske, *Platyhypnidium* M. Fleisch.). Species boundaries are thus based on differences in the gametophytes, which exhibit simple morphologies and are especially prone to plasticity and convergence. Such a reliance on a few, variable characters has led to unstable taxonomical systems, where species boundaries have varied as a function of the weight given to selected characters (e.g.,

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Hedenäs, 1995, and references therein). As a result, many aquatic moss species were shifted to different genera, families, or even higher lineages of mosses (e.g., *Vittia* Ochyra, Vanderpoorten et al., 2003a).

In this context, several authors attempted to clarify the systematics of troublesome taxa by a molecular phylogenetic approach and detected a severe conflict between morphology and molecules (e.g., Shaw & Allen, 2000). The taxonomic and evolutionary significance of such an incongruence cannot be assessed, though, as long as morphological variation has not been partitioned into a genetic and environmental component. A clearer picture of species delineation involves the sorting of the morphological variation due to plasticity in order to focus on morphological evolution to find stable, phylogenetically sound characters. Probably in part due to the difficulties of their experimental cultivation, aquatic mosses have, however, rarely been grown to address such problems.

In this paper, I will illustrate these issues by reviewing recent information on molecular phylogenetics and morphological evolution in *Hygroamblystegium*, one such troublesome moss genus that I have been involved with during the last years (Vanderpoorten et al., 2001, 2002a, b, 2003a, 2004; Vanderpoorten & Jacquemart, 2004), to finally discuss the possibilities of its systematic treatment and make the appropriate nomenclatural changes.

Previous Taxonomic Treatments of *Hygroamblystegium*: A Review and the Need to Define a Stable, Monophyletic Genus

In the context of a lack of a well-defined circumscription of the genus (contrast, e.g., Crum & Anderson, 1981; Frey et al., 1995; Hedenäs, 2003), phylogenetic analyses of nuclear and chloroplast sequence data were performed to redefine *Amblystegium* Schimper s.l. with respect to a revised concept of the family of the Amblystegiaceae (Vanderpoorten et al., 2001, 2002a, 2003a). The core *Amblystegium* species was found to be polyphyletic. It was proposed that *A. serpens* (Hedw.) Schimp., the type species, remains alone within a monotypic *Amblystegium* genus. The position of *A. riparium* (Hedw.) Schimp. in another genus, *Leptodictyum* (Schimp.) Warnst. closely related to *Campylium* (Sull.) Mitt. and *Campyliadelphus* (Kindb.) R. S. Chopra, was confirmed. The other species belong to *Hygroamblystegium* Loeske, a genus exhibiting a coarser morphology and a tendency to occur in wet or aquatic environments (Vanderpoorten et al., 2003a).

Within *Hygroamblystegium*, a morphologically ill-defined genus with a tendency to occur in wet or aquatic habitats, no less than 46 species have been included (Missouri Botanical Garden nomenclatural data base; ⟨http://mobot.mobot.org/W3T/Search/most.html⟩). Recent phylogenetic analyses, however, indicated that a number of taxa were erroneously included within the genus due to morphological convergence. For example, *H. tenax* var. *spinifolium* (Schimp.) Jenn. and *H. curvicaule* (Jur.) Loeske were both shown to belong to the genus *Cratoneuron* (Sull.) Spruce (Vanderpoorten et al., 2002b). *Hygroamblystegium macroneuron* Grout, a puzzling species only known from a few localities in Texas, has even been reinterpreted as a member of the Brachytheciaceae (Vanderpoorten et al., 2001, 2002b). In addition, extensive reductions to synonymy within the genus were recently published (e.g., Ochyra & Streimann, 2001). As a consequence, no fewer than 13 synonyms of *H. varium* (Hedw.) Mönk. were reported in a revision only concerning the Amblystegiaceae of southern Chile (Ochyra & Matteri, 2001). A few species are known only from the type locality and/or old herbarium collections (e.g., *H. calcareum* Kanda) and therefore precluded their inclusion in this study, which included the five widely recognized species: *H. fluviatile* (Hedw.) Loeske, *H. humile* (P. Beauv.) Vanderpoorten, Goffinet & Hedenäs, *H. noterophilum* (Sull. & Lesq.) Warnst., *H. tenax* (Hedw.) Jenn., and *H. varium*.

Using Phylogenies as a Template for Reconstructing Morphological Evolution

Hygroamblystegium species constitute a series of morphological intergrades, and conflicting taxonomic treatments arose from different interpretation of character significance, weight, and range (Table 1). In order to test previous concepts and investigate morphological evolution for reliable taxonomic characters that would discriminate *Hygroamblystegium* species, a species-level phylogeny was tentatively approached by analyzing sequence data from ITS, the most variable region currently widely used in bryophyte phylogenetics (Shaw et al., 2002), and two non-coding chloroplast regions (Vanderpoorten et al., 2004). Unfortunately, the level of molecular variability is extremely low in the Hypnales as compared to the other mosses (Shaw et al., 2003), and the level of variability found in the Amblystegiaceae is among the lowest within that order (unpublished data). As an example, the

Table 1. Examples of conflicting taxonomic circumscriptions of selected species included within *Hygroamblystegium*.

Taxon	Reference	Width of the costa at base (μm)	Length of the costa (in proportion of leaf length)	Laminal cells ratio
H. humile	Touw & Rubers (1989)	40–95	<2/3 to percurrent	(1.5–)2–3.5(–6)
	Smith (1978)	<40	<−3/4	2.5–5
	Crum & Anderson (1981)	—	<−2/3	3–6
	Hedenäs (2003)	31–63	50%–65%	3.25–6
	Frey et al. (1995)	—	<1/2	—
H. tenax	Touw & Rubers (1989)	55–120	percurrent to shortly excurrent	2–3(–4)
	Smith (1978)	50–60	percurrent to shortly excurrent	2–4
	Crum & Anderson (1981)	35–75	2/3 to percurrent	2–3
	Hedenäs (2003)	60–94.5	upper acumen to percurrent	3–6
	Frey et al. (1995)	—	percurrent	3–6
H. varium	Touw & Rubers (1989)	40–95	<2/3 → percurrent	(1.5–)2–3.5(–6)
	Smith (1978)	<40	>3/4	2–4(–4.5)
	Crum & Anderson (1981)	20–35	percurrent	2–3
	Hedenäs (2003)	29.5–58 (–77.5)	ending in acumen	2.5–6
	Frey et al. (1995)	—	percurrent	2–3

expected average (and standard deviation) number of substitution per site within the ITS region between species pairs in *Hygroamblystegium* and *Drepanocladus* (Müll. Hal.) G. Roth, calculated from available GenBank sequence data after selection of the substitution model best fitting each data set (Posada & Crandall, 1998), is about 0.0075 (0.0045) and 0.0066 (0.0046), respectively. As a comparison, these numbers reach up to 0.0583 (0.061) and 0.0632 (0.041), i.e., are about 10 times higher, in *Rhytidiadelphus* (Limpr.) Warnst. and *Anomodon* Hook. & Taylor, respectively. Consequently, the species-level phylogeny of *Hygroamblystegium* derived from the combined cp and ITS data set was mostly unsupported and unresolved. A screening of several nuclear genes showed that the gene coding for adenosine-kinase (*adk*) was polymorphic but, probably as a result of the high and variable polyploid states encountered within each *Hygroamblystegium* species (Fritsch, 1982), was present as multiple copies within individuals, making paralogy a problem for phylogenetic analyses (Vanderpoorten et al., 2004). To circumvent this issue, inter-simple sequence repeats (ISSR), a promising fingerprinting technique to detect molecular variation among closely related moss species (Vanderpoorten et al., 2003b; Werner et al., 2003), were used to obtain a tree of genetic relatedness that served as a backbone to investigate morphological evolution in the genus (Vanderpoorten & Jacquemart, 2004).

Partitioning Environmentally and Genetically Induced Morphological Variation for Investigating Morphological Evolution in *Hygroamblystegium*

Taxonomical significance of morphological characters that have traditionally been used for species delineation in *Hygroamblystegium* was tested by sorting out the plasticity in order to focus on morphological evolution, using comparative methods (Vanderpoorten & Jacquemart, 2004). Under controlled conditions, the total variance of almost all the characters substantially decreased. A number of features previously used for species delineation converged under identical growth conditions. For example, plants with an oval leaf shape and an obtuse leaf apex, matching the circumscription of *H. fluviatile*, eventually exhibited, after cultivation, triangular, acuminate leaves that are typical for *H. tenax* (Fig. 1). The ratio length of the costa to leaf length, mostly used to distinguish *H. humile*, was also very variable (Fig. 1).

Within the remaining, genetically fixed variation, using the tree of genetic relatedness to study morphological evolution indicated that the features that have been used for species delineation may not be appropriate for taxonomy (Fig. 2). Indeed, when the common path length between species pairs was set to 0 to simulate a scenario where common ancestry, i.e., phylogenetic history, would have no explanatory power on morphology, the likelihood of the data did not significantly decrease for many characters.

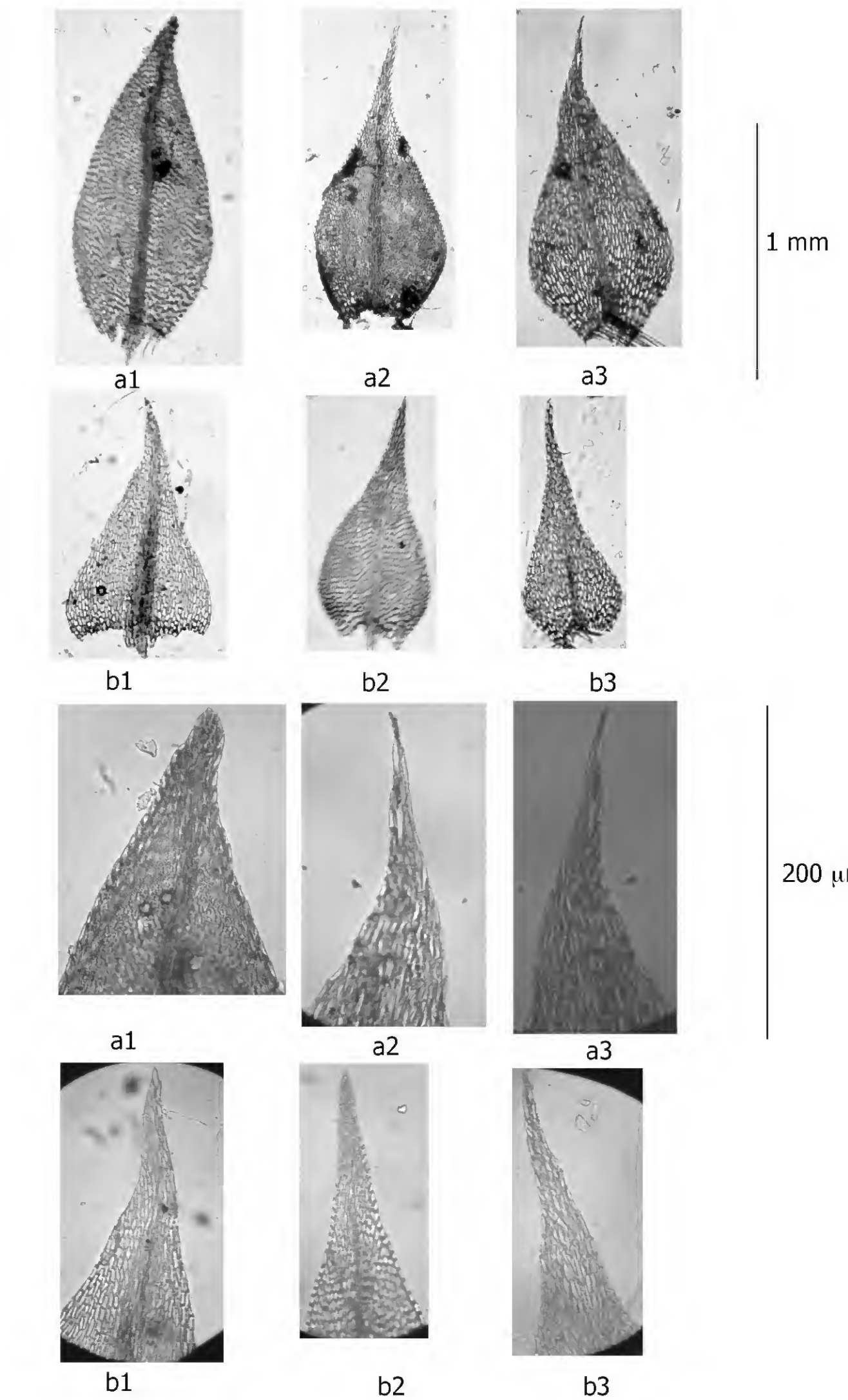

Figure 1. Plasticity of leaf shape (above) and apex morphology (below) in *Hygroamblystegium*. —a. Before cultivation. —b. After three months of cultivation under identical growth conditions. 1. *Vanderpoorten s.n.* (fast-flowing mountain brook in the Black Forest, Germany); 2. *Vanderpoorten s.n.* (weir on the Meuse River, Belgium); 3. *Vanderpoorten s.n.* (eutrophic marsh in Brussels, Belgium).

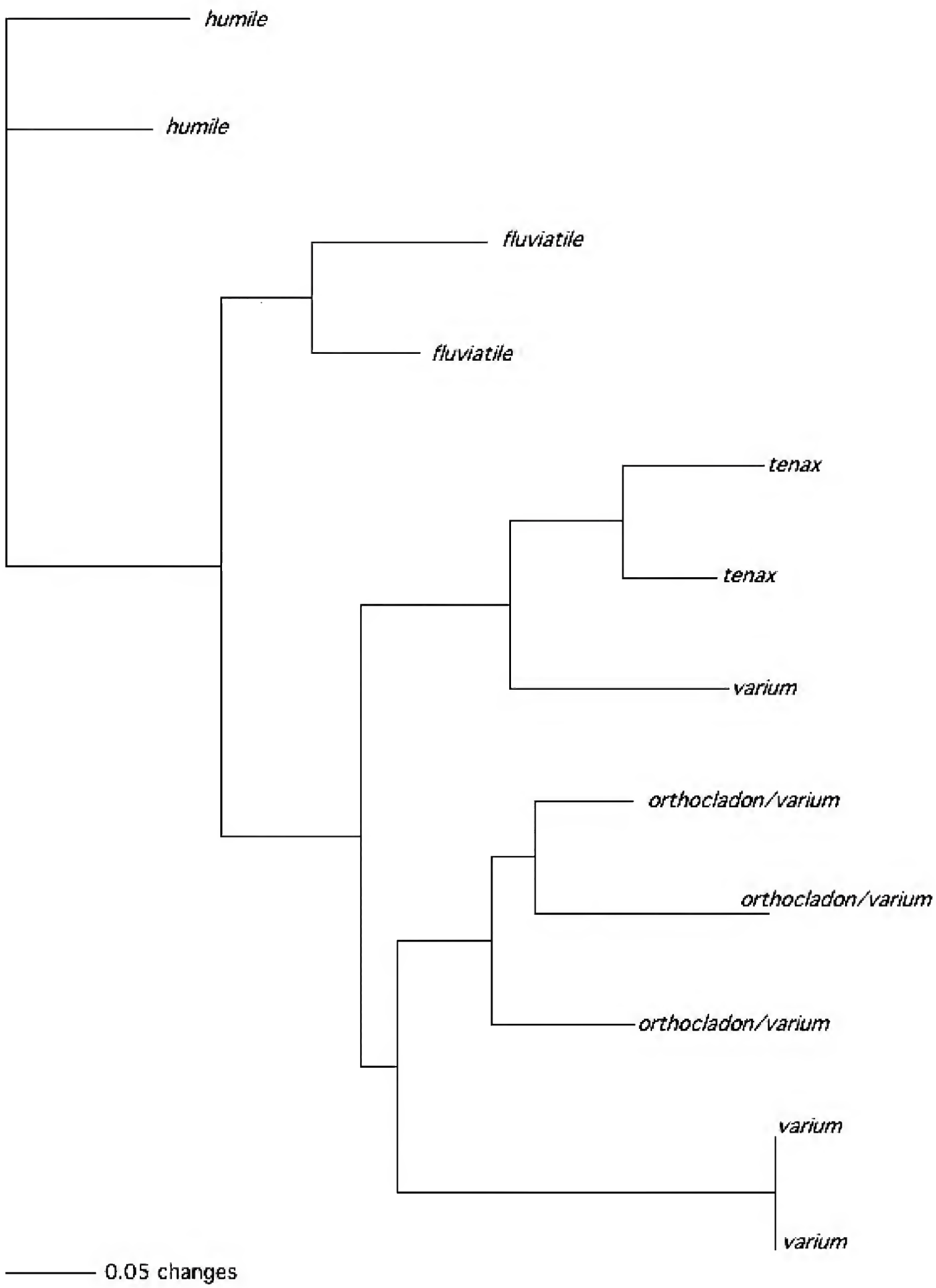

Figure 2. Neighbor-joining tree resulting from the analysis of 45 scorable ISSR fragments (redrawn from Vanderpoorten & Jacquemart, 2004). Each terminal node corresponds to 5 populations that exhibited identical ISSR patterns. Species names were based on examination of morphology before cultivation and were tentatively given based on Frey et al. (1995) Flora of European mosses, whose treatment conflicts with others (see Table 1). The taxa labelled as "orthocladon/varium" are especially problematic, as they share the characteristics of different species, including the possession of a significant proportion of obtuse leaf apices characteristic for *H. fluviatile* and *H. orthocladon*, included within *H. tenax* by Crum and Anderson (1981) but obviously conspecific with *H. fluviatile* (Flowers, 1973); 50–70 mm wide costae at base (excluding *H. varium* according to most treatments of Table 1); and short (1–2 : 1) laminal cells characteristic for the latter species.

In addition, performing asymmetrical transformations of branch length towards the most external and internal nodes, respectively, to simulate accelerated versus decelerated rates of evolution suggested that the evolution of most characters tended to accelerate towards the tips. As a consequence, the morphology of those taxa is likely to evolve rapidly and thus lacks the stability that would be required for species delineation. Finally, significant correlations were found between character pairs, many of which are related to plant size, suggesting the existence of evolutionary constraints. Thus, the morphological characters that have traditionally been used for species delineation in *Hygroamblystegium* lack the independence, diagnostic value for specific lineages, and stability that would be required for distinguishing different species.

Taxonomical Implications

Traditional *Hygroamblystegium* species do not fit with either a phenetic or phylogenetic species definition. Indeed, the characters that have been used to circumscribe species are either plastic or lack the stability and phylogenetic association that would be required to define species. Hence, *Hygroamblystegium* species as usually understood should be rejected. Can we propose an improved taxonomy for the genus based on studies of morphological evolution in a phylogenetic context? The identity of ISSR genome fingerprints among individuals of a same population (Vanderpoorten & Jacquemart, 2004) suggests that these populations could be clonal, which is consistent with the observations that these plants are rarely fertile. Clonal evolution of the genome would also explain the observed evolutionary patterns of *adk* within the genus, with ancestral gene duplication and independent evolution of paralogous copies (Vanderpoorten et al., 2004). The hypothesis of clonal evolution of different lineages is currently tested by using microsatellites, a marker that has recently been successfully used in the moss genus *Polytrichum* Hedw. to describe and explain patterns of genetic structure in relation to the breeding system (van der Velde et al., 2001a, b; Wilson & Provan, 2003). If this hypothesis is verified, the situation in *Hygroamblystegium* would parallel that encountered in complex of agamosperm vascular plants, such as *Taraxacum* F. H. Wigg. or *Rubus* L., where the wide range of genetic variability within "species" of polyphyletic origin is kept due to the fast and independent evolution of polyploid genomes (see Gornall, 1999, for a review) and is unlikely to be associated with any kind of pattern of morphological evolution. In this case, I would pragmatically suggest that all the *Hygroamblystegium* species should be merged into a single species. Thus, *Hygroamblystegium* would consist of a single species with a wide range of morphologies and ecologies, from tree boles in forest to fast-flowing streams, which is actually a fairly common case in other widespread moss species such as, for example, *Brachythecium ruderale* (Brid.) W. R. Buck, *Ceratodon purpureus* (Hedw.) Brid., *Cratoneuron filicinum* (Hedw.) Spruce, or *Dicranum scoparium* Hedw.

Nomenclatural Changes Involved

Hygroamblystegium varium is, with *H. fluviatile* and *H. tenax,* the first described species of the genus as currently circumscribed. The name *H. varium* is retained here because it is especially appropriate to describe the range of uncorrelated morphologies, ecologies, and genotypes found in the species. A lectotype of *Hypnum noterophilum* is designated here because Sullivant (1856) based his original description of the species on two literature citations: (1) James (1855, p. 417, reporting a specimen collected by Porter in limestone springs, Franklin Co., Pennsylvania), and (2) Sullivant & Lesquereux (1857, N° 348: "Hab. in rivulis calcariis Pennsylvaniae (cl. Porter); et locis similibus Wisconsin"). Sheet 348 of "Musci Boreali-Americani" includes two specimens, but without any indication on their origin. The left specimen of that sheet is selected here as lectotype. Indeed, its similarity with a specimen in ILL, subsequently collected by Porter and labeled as follows: "Musci of Pennsylvania by Thomas P. James—1871. Amblystegium noterophilum S. & L. Limestone springs. Near Mercesburg Franklin Co. (T. C. Porter)," suggests that it may correspond to Porter's Pennsylvanian collection. The right specimen on the sheet of N° 348 of "Musci Boreali-Americani" is a syntype. It is similar to a specimen in FH collected by Lesquereux in Wisconsin and labeled as follows: "Hypnum irriguum v. spinifolium Sch. H. noterophilum Sulliv. et Lesq. Wisconsin Leg. Lesquereux."

The following synonymies are introduced:

Hygroamblystegium varium (Hedw.) Mönk., Hedwigia 50: 275. 1911. Basionym: *Leskea varia* Hedw., Sp. Musc. Frond.: 216. 1801. Type: U.S.A. Pennsylvania: Lancaster, s.d., *Mühlenberg s.n.* (lectotype, G!, designated by Hedenäs & Geissler, 1999).

Hygroamblystegium fluviatile (Hedw.) Loeske, Moosfl. Harz: 299. 1903, syn. nov. Basionym:

Hypnum fluviatile Hedw., Sp. Musc. Frond.: 277. 1801. TYPE: Sweden. s.d., *Swartz s.n.* (lectotype, G, designated by Hedenäs & Geissler, 1999).

Hygroamblystegium humile (P. Beauv.) Vanderpoorten, Goffinet & Hedenäs, Plant Syst. Evol. 241: 7. 2003, syn. nov. Basionym: *Hypnum humile* P. Beauv., Prodr. Aethéogam.: 65. 1805. TYPE: U.S.A. Pennsylvania: s.d., *s.n.* (isotype, S, separated from the Arnott material in GL (Crundwell, 1981)).

Hygroamblystegium noterophilum (Sull. & Lesq.) Warnst., Krypt. Fl. Mark. Brandenb., vol. 2: 890. 1906. Basionym: *Hypnum noterophilum* Sull. & Lesq. in Sull., Musci Hepat. U.S. (Repr.): 78. 1856 (X). TYPE: U.S.A. Pennsylvania: Franklin County, s.d., T. C. Porter *s.n.* (lectotype, FH!, designated here by R. Ochyra; isotypes: ILL!, NY!, VT-Frost!, VT-Pringle!, WIS!, WTU!).

Hygroamblystegium tenax (Hedw.) C. Jens., Skand. Bladm.: 483. 1939, syn. nov. Basionym: *Hypnum tenax* Hedw., Sp. Musc. Frond.: 277. 1801. TYPE: U.S.A. Pennsylvania: Lancaster, s.d., *Mühlenberg s.n.* (lectotype, G, designated by Hedenäs & Geissler, 1999).

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PHYLOGENY AND EVOLUTION OF EPIPHYTISM IN THE THREE MOSS FAMILIES METEORIACEAE, BRACHYTHECIACEAE, AND LEMBOPHYLLACEAE[1]

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Abstract

The phylogeny of three pleurocarpous moss families, Meteoriaceae, Brachytheciaceae, and Lembophyllaceae, was studied using both morphology and molecular data. Phylogenetic analyses using different approaches revealed very similar, well-resolved topologies wherein the Meteoriaceae and Brachytheciaceae are monophyletic and sister to each other. The Lembophyllaceae were found not to be closely related to those families, but to the Neckeraceae. Results suggest several independent origins of epiphytism within these families. Character states that are commonly regarded as adaptations to epiphytism are concentrated on those clades with epiphytic species. The evolution of certain characters, such as peristomes that open under humid conditions, seta length, and spore size, is clearly correlated with an epiphytic habit. In phylogenetic analyses, this kind of morphological character could be suspected to lead to biased results if species are grouped based on shared ecological conditions instead of common ancestry. Our study revealed that despite correlated evolution with epiphytism, these characters do not significantly deteriorate the robustness of the hypotheses on relationships between families. However, there may be negative effects on the phylogenetic reconstruction by increasing homoplasy, as well as incongruence between data partitions, and decreasing the phylogenetic structure obtained with morphological data.

Key words: adaptation, Brachytheciaceae, correlated evolution, direct optimization, epiphytism, Lembophyllaceae, Meteoriaceae, morphological evolution, phylogeny.

For a long time bryologists have suspected that some of the morphological character states common in epiphytic mosses are the products of convergent evolution into similar habitats (Grout, 1908; Patterson, 1953; Vitt, 1981; Buck & Vitt, 1986; Buck, 1991; Hedenäs, 1998, 2001). These include both sporophytic characters, such as an upright capsule, a reduced peristome with weak hygrocastique movements (see Neumann & Mueller, 1987), large spores, and a short seta, as well as gametophytic ones, such as pendent shoots and papillose leaf cells. In the sporophytic generation, epiphytism seems to lead to a reduction of several features. This is probably related to improved spore dispersal due to growing high up in trees as compared to terricolous species, the sporophytes of which rarely exceed five centimeters in height. In epiphytic species, for example, the seta length and hygroscopic movements of the peristome may not play as an important role in successful dispersal. It has been suggested that hygrocastique peristomes are advantageous for epiphytes that are dependent on rarely occurring moist conditions, allowing the germination of dispersed spores (Hedenäs, 1998). The connection between large spores and epiphytic habitats was explained by Hedenäs (2001) as an adaptation to relatively long-lasting microhabitats that are often occupied by species with the long-lived shuttle life strategy (During, 1979). In the gametophyte generation, most of the character states commonly found in epiphyte species are shared with those in xeric habitats and hence related to retention of wa-

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ter. Examples of this include the lack of a central strand in the stems, papillose leaf cells, attenuate leaf apices, as well as thick cell walls (Vitt, 1981; Hedenäs, 2001).

Despite the prevailing practice among bryologists to refer to these morphological modifications as adaptations, it is uncertain whether these characters represent true adaptations for epiphytic habitats. Widely used definitions of adaptation stress the role of natural selection and fitness differences between phenotypes in evolution of adaptive characters (Gould & Vrba, 1982; Coddington, 1988). In epiphytic mosses, however, evidence for fitness differences between adaptive and non-adaptive phenotypes and the role of selection in their evolution is lacking. Especially in the case of morphological reductions, these modifications might as well result from relaxed ecological constraints enabling the simplification of complex characters (see Wilkens, 1988; Fong et al., 1995). Some of the possible adaptations in epiphytic mosses may also turn out to be exaptations (Gould & Vrba, 1982), i.e., characters that increase the fitness in epiphytic species, but not due to evolution that is connected to epiphytism. While demonstrating the adaptive nature of morphological characters in a given environment is a complex task, several methods have been developed for detecting correlated evolution of selected characters (e.g., Maddison, 1990; Pagel, 1994). These methods enable the recognition of the most promising candidates among all characters for further studies on adaptive evolution.

If very similar morphological modifications are present in several unrelated organisms only due to shared habitat preferences, this has a potential to hamper the phylogenetic analyses. Characters correlated with epiphytism may cause problems in the phylogenetic analyses of pleurocarpous mosses, which has already been discussed by Hedenäs (1998) and Newton and DeLuna (1999). If the phylogenetic signal in this kind of "ecological characters" conflicts with other characters, including them in phylogenetic analyses could lead either to increased overall homoplasy and a less resolved phylogeny or to biased results reflecting similar ecological preferences instead of shared phylogenetic history. In addition, the correlated evolution of characters leads to problems of independence of characters, which is one of the basic assumptions in phylogenetic analyses (Kluge, 1989). Therefore, the use of these characters, or sometimes even morphological data in general, is rejected in favor of molecular data (McCracken et al., 1999). On the other hand, the exclusion of some parts of data prior to the analyses will also in any situation lead to eliminating part of the phylogenetic signal from analyses, which may affect the obtained hypothesis of relationships (e.g., Kluge, 1989; De Queiroz, 1996).

In this study we concentrate on three pleurocarpous families, the Meteoriaceae, Brachytheciaceae, and Lembophyllaceae. In two of them, the Meteoriaceae and Lembophyllaceae, the majority of species are epiphytic. In the Brachytheciaceae, most of the species grow in the forest floor layer on rocks, wood, and soil, or on cliffs. Whereas the Meteoriaceae and Lembophyllaceae are most common in tropical areas of the world, the Brachytheciaceae are most diverse in cool-temperate and boreal regions of the Northern Hemisphere. We aim at resolving the phylogenetic relationships among these three families using both morphological and DNA data. We compare two cladistic methods, direct optimization (Wheeler, 1996) and parsimony ratchet (Nixon, 1999a) analyses of manually aligned data. The obtained phylogenies were then used to study the evolution of epiphytism, with special attention to characters that are suspected to have an adaptive value in epiphytic species. Because the convergent evolution of these characters may lead to biased conclusions on phylogenetic relationships, we further investigate whether evolution of the morphological characters used in phylogenetic analyses correlates with epiphytism and evaluate their effect on phylogenetic reconstruction.

Materials and Methods

Selection of Taxa and Morphological Data

Because relationships between hypnalean moss families (Hypnales s.l.) are still rather poorly known (Buck et al., 2000a), we included several species outside the Meteoriaceae, Brachytheciaceae, and Lembophyllaceae (Table 1). Whenever material was available, the type species were used as an exemplar.

A morphological data matrix was compiled using mainly herbarium specimens available in H and from recent collections of the Brachytheciaceae and Meteoriaceae from Hunan, China (Ignatov et al., 2004; Huttunen, unpublished data). The morphological data include information of 16 sporophytic and 44 gametophytic characters. The information on morphology was initially based on the same herbarium specimens that were used for sequencing, but in some cases it was supplemented using other specimens belonging to same the morphotype as the sequenced specimen. For two taxa, *Meteorium subpolytrichum* and *Floribundaria pseudofloribunda*, sporophytic characters were obtained from the lit-

erature (see Appendix 1). Morphological characters, their states, and explanations are given in Appendix 1. The complete character matrix is given in Appendix 2.

DNA EXTRACTION, PCR, AND SEQUENCING

Protocols for DNA extraction, PCR, and sequencing follow the standard methods used in earlier studies by the authors (Huttunen & Ignatov, 2004; Quandt et al., 2004b). A list of all included species, voucher specimens, and GenBank accession numbers is provided in Table 1.

PHYLOGENETIC ANALYSES

We tested the effect of the alignment method on the resulting topology, and data were analyzed using two different methods: (1) a direct optimization (Wheeler, 1996) as employed in the program POY (Gladstein & Wheeler, 2001), and (2) parsimony ratchet analyses (Nixon, 1999a) of manually aligned sequence data with PAUP* (Swofford, 2002) and PRAP (Müller, 2004).

We did POY analyses using parallel computing of eight processors of Silicon Graphics Origin 2000/128 hardware in the Center of Scientific Computing, Espoo, Finland. In order to save computation time and memory, longer sequences were cut into shorter fragments and then submitted to POY (Gladstein & Wheeler, 2001). Commands used in all POY analyses are given in Appendix 3. POY utilizes indel information during phylogeny reconstruction, and in our analyses gaps obtained the same weight as all other substitution events. In direct optimization both alignment and phylogenetic analyses are performed simultaneously by optimizing all characters onto the tree using the up- and down-pass optimization methods (see also Schulmeister et al., 2002). We used parsimony as an optimality criterion in direct optimization, hence the character optimizations ("alignments") and topologies with the smallest number of evolutionary changes were selected as the best hypothesis for relationships within the study group.

Both jackknife and Bremer support for clades in the most parsimonious POY trees were obtained by analyzing POY character optimizations as static alignments in Nona (Goloboff, 1994) within the Winclada shell (Nixon, 1999b). Jackknife support was calculated with 1000 replicates of 10 random addition cycles each. Bremer support values (Bremer, 1994) were calculated by saving suboptimal trees up to 10 steps longer. Bremer support analyses included 500 random addition sequence replicates, holding 20 trees in each replicate for TBR branch swapping and finally retaining a maximum of 10,000 trees.

Based on the criteria laid out in Kelchner (2000) and Borsch et al. (2003), DNA sequences were manually aligned using the alignment editor Align (Hepperle, 1997). Parsimony analyses including only molecular data were performed using PRAP (Müller, 2004), running parsimony ratchet analyses within PAUP* 4.0b10 (Swofford, 2002). In the parsimony ratchet 10 random addition cycles of 200 iterations were employed using default settings. Branch support was estimated by 500 cycles of parsimony jackknifing, applying 50 ratchet iterations per cycle and using PRAP. Further measurement of support (Bremer support; Bremer, 1994) for the individual clades was also obtained using PRAP (Müller, 2004).

The data partitions (*psb*T-H, *trn*L-F, ITS2, and morphology) were analyzed independently and in combination with POY and PAUP/PRAP (see Table 2). The tree lengths from all these analyses were used for calculating incongruence length differences (ILD; Mickevich & Farris, 1981; Farris et al., 1995).

MORPHOLOGICAL CHARACTER EVOLUTION AND THE EFFECT OF CORRELATED CHARACTERS ON PHYLOGENY RECONSTRUCTION

To study if the evolution of particular morphological characters correlates with epiphytism, we performed the concentrated changes test (CCT; Maddison, 1990). All characters were first transformed into binary characters, yielding 64 characters to test (Table 3). Only clades containing all members of the Brachytheciaceae, Meteoriaceae, and Lembophyllaceae (BML clade in Fig. 2) were included in CCT excluding most of the outgroup taxa with slightly higher variation in branch lengths. For the ancestral state reconstruction, accelerated and delayed character state transitions were tested. The CCT was performed using MacClade 3.05 (Maddison & Maddison, 1992) with 10,000 simulations based on the actual number of character state changes, with either one of the character states allowed as ancestral state for the dependent character at the base of the tree. For epiphytism two different definitions were applied: (1) in the "strict definition" (P^1 in Table 3; species marked with asterisk in Fig. 4), only those species were accepted as epiphytes that exclusively grow on tree trunks, branches, twigs, shrubs, or bushes; and (2) in a slightly broader definition those species were accepted as belonging to the epiphyte group that occasionally are found in other above ground

Table 1. GenBank accessions and voucher specimens for sequenced specimens. Voucher specimens are in H if not otherwise indicated. Of those specimens that are held in Moscow (MHA), duplicates are also present in H.

Taxon	Voucher specimen	GenBank accession No.		
		*trn*L-F	*psb*T-H	ITS2
Brachytheciaceae				
Aerolindigia capillacea (Hornsch.) M. Menzel	Ecuador, *D. H. Norris 92175 & M. Bolivar*	AY044072	AF417395	AF395634
Aerobryum speciosum (Dozy & Molk.) Dozy & Molk.	China, *T. Koponen et al. 52753*	AF397780	AF417386	AF403619
Brachytheciastrum collinum (Müll. Hal.) Ignatov & Huttunen	Russia, 7 Aug. 1999, *M. Ignatov s.n.* (MHA)	AY184776	AY184757	AY166440
Brachytheciastrum falcatulum (Broth.) Ignatov & Huttunen	Russia, *M. Ignatov #0/1680* (MHA)	AF397774	AF417468	AF403662
Brachytheciastrum velutinum (Hedw.) Ignatov & Huttunen	Finland, 23 Aug. 1988, *Kiirikki s.n.*	AF397832	AF417440	AF403667[2]
Brachythecium acuminatum (Hedw.) Austin	U.S.A., *L. E. Anderson 24257*	AF397864	AF417442	AF403654
Brachythecium albicans (Hedw.) Schimp.	Finland, *J. Pykälä 6775*	AF397875	AF417444	AF403646
Brachythecium buchananii (Hook.) A. Jaeger	China, *T. Koponen et al. 53972*	AF397792	AF417348	AF403595
Brachythecium cirrosum (Schwägr.) Schimp.	China, *B. C. Tan 93-846*	AF397856	AF417449	AF403641
Brachythecium complanatum Broth.	Russia, *M. Ignatov 97-172* (MHA)	AY184781	AY184762	AY166444
Brachythecium erythrorrhizon Schimp. ssp. *asiaticum* Ignatov	Russia, *M. Ignatov 97-1120* (MHA)	AY184783	AY184763	AY166446
Brachythecium frigidum (Müll. Hal.) Besch.	U.S.A., 23 Apr. 1981, *Duell s.n.*	AF397874	AF417456	AF403638
Brachythecium geheebii Milde	Russia, *E. Ignatova #15/68* (MHA)	AF397776	AF417467	AF403660
Brachythecium glaerosum (Spruce) Schimp.	Ukraina, 13 Sep. 1991, *M. Ignatov s.n.* (MHA)	AF397865	AF417457	AF403656
Brachythecium laetum (Brid.) Schimp.	U.S.A., *B. Allen 6208*	AY184782	—	AY166445
Brachythecium lamprocarpum (Müll. Hal.) A. Jaeger	Papua New Guinea, *T. Koponen 28748*	AF397806	AF417394	AF403584
Brachythecium mildeanum (Schimp.) Schimp.	Russia, 20 May 1999, *M. Ignatov s.n.* (MHA)	AY184777	AY184758	AY166441
Brachythecium rivulare Schimp.	Finland, 19 May 1996, *A. Parnela s.n.*	AF397866	AF417460	AF403651
Brachythecium ruderale (Brid.) W. R. Buck	Haiti, *W. R. Buck 4826*	AY184784	AY184764	AF503535
Brachythecium rutabulum (Hedw.) Schimp.	Finland, *S. Huttunen 1415*	AF397867	AF417447	AF403644
Brachythecium salebrosum (F. Weber & D. Mohr) Schimp.	Finland, *S. Laaka 1835*	AF397857	AF417448	AF403648
Brachythecium salebrosum (F. Weber & D. Mohr) Schimp.	Russia, 15 Aug. 1996, *M. Ignatov s.n.* (MHA)	AY184780	AY184761	AY16643
Brachythecium subalbicans Broth., nom. illeg.	China, *T. Koponen et al. 49112*	AF397788	AF417373	AF403583
Brachythecium tommasinii (Boulay) Ignatov & Huttunen	Poland, 26 Aug. 1982, *R. Ochyra s.n.*	AF397870	AF417464	AF403639[1]
Brachythecium turgidum (Hartm.) Kindb.	Russia, *M. Ignatov 31/291* (MHA)	AY184785	AY184765	AY166447
Brachythecium udum I. Hagen	Russia, *M. Ignatov 00-843* (MHA)	AY184778	AY184759	AF503536
Bryhnia scabrida (Lindb.) Kaurin	Russia, *S. Popov 26* (MHA)	AF397826	AF417405	AF403588
Bryoandersonia illecebra (Hedw.) H. Rob.	U.S.A., *Bowers 22214*	AF397819	AF417365	AF403626
Cirriphyllum crassinervium (Taylor) Loeske & M. Fleisch.	Russia, 23 Aug. 1999, *M. Ignatov s.n.* (MHA)	AF397868	AF417433	AF403668[2]
Cirriphyllum koponenii (Ignatov) Ignatov & Huttunen	Papua New Guinea, *T. Koponen 32122*	AF397872	AF417446	AF403657
Cirriphyllum piliferum (Hedw.) Grout	Finland, *T. Koponen & S. Huttunen 1324*	AF397799	AF417403	AF403608
Clasmatodon parvulus (Hampe) Sull.	U.S.A., 23 Apr. 1992, *P. L. Redfearn Jr. & B. Allen s.n.*	AF397813	AF417430	AF403614

Table 1. Continued.

Taxon	Voucher specimen	GenBank accession No.		
		*trn*L-F	*psb*T-H	ITS2
Donrichardsia macroneuron (Grout) H. A. Crum & L. E. Anderson	U.S.A., *Redfearn 27208* (sequences from GenBank, Vanderporten et al., 2002	AY009848	—	AF167350
Donrichardsia patulifolia (Cardót & Thér.) Ignatov & Huttunen	China, *T. Koponen et al. 53920*	AF397850	AF417443	—
Eurhynchiastrum pulchellum (Hedw.) Ignatov & Huttunen	Finland, *T. Koponen & S. Huttunen 1321*	AY044069	AF417384	AF395635
Eurhynchiadelphus eustegium (Besch.) Ignatov & Huttunen	China, *T. Koponen 36592*	AF397790	AF417378	AF403602
Eurhynchium angustirete (Broth.) T. J. Kop.	Russia, 3 Jul. 1998, *M. Ignatov s.n.* (MHA)	AF397825	AF417436	AF403621
Eurhynchium striatum (Hedw.) Schimp.	Russia, 13 Aug. 1998, *Makridin s.n.* (MHA)	AY184788	AY184769	AF503538
Helicodontium capillare (Hedw.) A. Jaeger	Colombia, 4 May 1990, *S. Churchill et al. s.n.*	AF397855	AF417435	—
Homalotheciella subcapillata (Hedw.) Broth.	U.S.A., 29 Mar. 1995, *B. Allen & P. L. Redfearn Jr. s.n.* (NY)	AF397860	AF417462	AF403658
Homalothecium aureum (Spruce) H. Rob.	Turkmenia, 22 Nov. 1983, *I. B. Sirotina & A. Abramova s.n.* (MHA)	AF397876	AF417463	AF403650
Homalothecium fulgescens (Mitt.) Lawton	Canada, *H. Streimann 43136*	AF397877	AF417466	AF403637
Homalothecium laevisetum Sande Lac.	China, *T. Koponen et al. 54066*	AF397820	AF417351	AF403616
Homalothecium lutescens (Hedw.) H. Rob.	Czech Republic, 30 Apr. 1999, *J. Vana & J. Enroth s.n.*	AF397827	AF417379	AF403642
Homalothecium megaptilium (Sull.) H. Rob.	U.S.A., *A. Newton 5206* (BM)	AF397842	AF417431	AF403597
Homalothecium nuttallii (Wilson) A. Jaeger	U.S.A., 5 Aug. 1989, *M. Ignatov s.n.* (MHA)	AF397798	AF417399	AF403589
Homalothecium philippeanum (Spruce) Schimp.	Georgia, *V. Vasak B-90123* (MHA)	AF397863	AF417451	AF403647
Homalothecium sericeum (Hedw.) Schimp.	Finland, *T. Koponen & S. Huttunen 1322*	AF397805	AF417349	AF403587
Kindbergia praelonga (Hedw.) Ochyra	China, *T. Koponen et al. 50467*	AF397804	AF417385	AF403591
Meteoridium remotifolium (Müll. Hal.) Manuel	Mexico, *A. Newton 4399* (BM)	AF397833	AF417418	AF403601
Myuroclada maximowiczii (G. G. Borshch.) Steere & W. B. Schofield	China, *T. Koponen et al. 53505*	AF397779	AF417392	AF403625
Okamuraea brachydictyon (Cardót) Nog.	China, *T. Koponen et al. 48969*	AY184789	AY184771	AF503537
Oxyrrhynchium hians (Hedw.) Loeske	China, *T. Koponen et al. 53740*	AF397815	AF417372	AF403603
Oxyrrhynchium pumilum (Wilson) Loeske	Georgia, 8 Aug. 1987, *M. Ignatov s.n.* (MHA)	AY184790	AY184772	—
Oxyrrhynchium savatieri Besch.	China, *T. Koponen et al. 51775*	AF397859	AF417453	AF403659
Oxyrrhynchium vagans (A. Jaeger) Ignatov & Huttunen	Papua New Guinea, *T. Koponen 34413*	AF397862	AF417450	AF403652
Palamocladium euchloron (Müll. Hal.) Wijk & Margad.	Russia, 23 Aug. 1999, *M. Ignatov s.n.* (MHA)	AF397851	AF417416	AF403623
Palamocladium leskeoides (Hook.) E. Britton	China, *T. Koponen et al. 52596*	AF397791	AF417387	AF403596
Platyhypnidium austrinum (Hook. f. & Wilson) M. Fleisch.	Australia, *H. Streimann 49544*	AY184791	AY184773	AY166449
Platyhypnidium riparioides (Hedw.) Dixon	China, *T. Koponen et al. 51843*	AF397847	AF417411	—
Plasteurhynchium striatulum (Spruce) M. Fleisch.	Georgia, 1 Aug. 1987, *M. Ignatov s.n.* (MHA)	AY184792	AY184774	AY166450
Pseudoscleropodium purum (Hedw.) M. Fleisch.	Czech Republic, 30 Apr. 1999, *J. Vana & J. Enroth s.n.*	AF397797	AF417470	AF403663
Remyella brachypodia (M. Fleisch.) Ignatov & Huttunen	Papua New Guinea, *T. Koponen 33007*	AF397854	AF417423	AF403600

Table 1. Continued.

Taxon	Voucher specimen	GenBank accession No.		
		*trn*L-F	*psb*T-H	ITS2
Rhynchostegiella macilenta (Renauldt & Cardót) Cardót	Portugal, L. *Hedenäs* (B4503; S)	AF397781	AF417370	AF403570
Rhynchostegiella tenella (Dicks.) Limpr.	Georgia, 2 Oct. 1997, *M. Ignatov s.n.* (MHA)	AY044070	AF417400	AF395633
Rhynchostegiella teneriffae (Mont.) Dirkse & Bouman	Portugal, *S. Fontinha et al.* (B9121; S)	AF397783	AF417368	AF403569
Rhynchostegium arcticum (I. Hagen) Ignatov & Huttunen	Russia, *A. G. Bezgodov 222* (MHA)	AF397775	AF417424	AF403661
Rhynchostegium confertum (Dicks.) Schimp.	Georgia, 27 Aug. 1987, *M. Ignatov s.n.* (MHA)	AF397837	AF417413	AF403622
Rhynchostegium murale (Hedw.) Schimp.	Russia, 9 Jul. 1993, *E. Baisheva s.n.* (MHA)	AF397816	AF417355	AF403624
Rhynchostegium pallidifolium (Mitt.) A. Jaeger	China, *T. Koponen et al. 51301*	AF397807	AF417371	AF403618
Rhynchostegium psilopodium Ignatov & Huttunen	China, *T. Koponen et al. 51803*	AF397861	AF417452	AF403643
Rhynchostegium rotundifolium (Brid.) Schimp.	Russia, 31 Aug. 1999, *V. Onipchenco s.n.* (MHA)	AF397809	AF417396	AF403611
Rhynchostegium serrulatum (Hedw.) A. Jaeger	U.S.A., 12 Sep. 1992, *B. C. Tan s.n.* (MHA)	AF397829	AF417380	AF403620
Scleropodium obtusifolium (Mitt.) Kindb.	U.S.A., 13 Aug. 1989, *M. Ignatov & D. H. Norris s.n.*	AF397793	AF417434	AF403615
Sciurohypnum flotowianum (Sendtn.) Ignatov & Huttunen	Poland, 8 Jun. 1985, *H. & S. Bednarek s.n.* (MHA)	AF397869	AF417461	AF403636
Sciurohypnum glaciale (Schimp.) Ignatov & Huttunen	Russia, 8 Aug. 1986, *E. Ignatova s.n.* (MHA)	—	AY184766	AY166448
Sciurohypnum hylotapetum (B. L. Higinb. & N. L. Higinb.) Ignatov & Huttunen	Canada, *R. Love & J. Rogers B27688*	AF397871	AF417445	AF403653
Sciurohypnum latifolium (Kindb.) Ignatov & Huttunen	Russia, 31 Jul. 1998, *M. Ignatov s.n.* (MHA)	AY184786	AY184767	AF503534
Sciurohypnum oedipodium (Mitt.) Ignatov & Huttunen	Finland, *S. Huttunen 1439*	AF397784	AF417388	AF403649
Sciurohypnum ornellanum (Molendo) Ignatov & Huttunen	Russia, *M. Ignatov 16/44* (MHA)	AF397785	AF417354	AF403593
Sciurohypnum plumosum (Hedw.) Ignatov & Huttunen	China, *T. Koponen et al. 56777*	AF397814	AF417359	AF403586
Sciurohypnum populeum (Hedw.) Ignatov & Huttunen	Finland, *J. Pykälä 7029*	AF397873	AF417458	AF403640
Sciurohypnum reflexum (Stark.) Ignatov & Huttunen	Finland, *S. Huttunen 1195*	AF397858	AF417459	AF403655
Scorpiurium circinatum (Brid.) M. Fleisch. & Loeske	Georgia, 8 Aug. 1987, *M. Ignatov s.n.* (MHA)	AF379834	AF417410	AF403598
Scorpiurium deflexifolium (Solms) M. Fleisch. & Loeske	Portugal, *L. Hedenäs & I. Bisang (B22455; S)*	AF397844	AF417407	AF403599
Squamidium brasiliense (Hornsch.) Broth.	*Tanzania, T. Pócs et al. 88161/R*	AY044063	AF417393	AF395637
Unclejackia longisetula (E. B. Bartram) Ignatov, T. J. Kop. & D. H. Norris	Papua New Guinea, *T. Koponen 32496*	AF397794	AF417357	AF395643
Zelometeorium patulum (Hedw.) Manuel	Ecuador, *D. H. Norris et al. 91902*	AF397787	AF417362	—
Lembophyllaceae				
Bestia longipes (Sull. & Lesq.) Broth.	U.S.A., *D. H. Norris 82503*	AF397802	AF417401	AF403628
Camptochaete arbuscula (Sm.) Reichardt	New Zealand, *J.-P. Frahm 14-5* (J.-P. Frahm, CHR)	AF187250 and AF187266	—	—
Camptochaete arbuscula (Sm.) Reichardt	New Zealand, *H. Streimann 51408*	—	AF417356	AF403629
Dolichomitriopsis diversiformis (Mitt.) Nog.	Russia, 18 Jul. 1990, *V. A. Nedoluzhko s.n.* (MHA)	AF397777	AF417469	AF403664
Isothecium alopecuroides (Dubois) Isov.	Finland, *T. Koponen & S. Huttunen 1320*	AY044065	AF417353	AF395636
Lembophyllum clandestinum (Hook. f. & Wilson) Lindb.	New Zealand, *D. H. Vitt 29644*	AF397823	AF417374	AF403630
Noebarbella comes (Griff.) Nog.	China, *T. Koponen et al. 50975*	AY044066	AF417402	AF395628
Weymouthia mollis (Hedw.) Broth.	New Zealand, 24 Apr. 1999, *S. Zank s.n.* (W. Frey, D. Quandt, CHR)	AF187246 and AF187262	AF397885	AF188052
Meteoriaceae				
Aerobryidium filamentosum (Hook.) M. Fleisch.	Papua New Guinea, *T. Koponen 37908*	AF397789	AF417347	AF403613

Table 1. Continued.

Taxon	Voucher specimen	GenBank accession No.		
		*trn*L-F	*psb*T-H	ITS2
Aerobryopsis wallichii (Brid.) M. Fleisch.	Taiwan, *I. Ahonen et al. 1684*	AF397835	AF417437	—
Ancistrodes genuflexa (Müll. Hal.) Crosby	Chile, *R. Blöcher 34* (R. Blöcher)	AY101380	AF509936	AF509837
Barbella flagellifera (Cardót) Nog.	China, *T. Koponen et al. 56995*	AY044071	AF417360	AF395630
Barbella flagellifera (Cardót) Nog.	Japan, *M. Mizutani 15609* (J.-P. Frahm)	AF509547	AF397882	AF509844
Barbellopsis trichophora (Mont.) W. R. Buck	China, *T. Koponen et al. 57394*	AY044062	AF417363	AF395625
Chrysocladium retrosum (Mitt.) M. Fleisch.	China, *T. Koponen et al. 55572*	AY044061	AF417390	AF395626
Cryptopapillaria fuscescens (Hook.) M. Menzel	Philippines, 26 Apr. 1991, *B. C. Tan s.n.*	AY044064	AF417404	AF395638
Cryptopapillaria penicillata (Dozy & Molk.) M. Menzel	Bolivia, *U. Drehwald 40175* (Drehwald)	AF509553	AF509948	AF509850
Diaphanodon blandus (Harv.) Renauld & Cardót	Philippines, 29–30 Nov. 1986, *B. C. Tan s.n.*	AF397830	AF417364	AF403605
Duthiella wallichii (Mitt.) Müll. Hal.	China, *T. Koponen et al. 54168*	AF397782	AF417375	AF403612
Floribundaria floribunda (Dozy & Molk.) M. Fleisch.	Papua New Guinea, *T. Koponen 35719*	AF397800	AF417428	AF403580
Floribundaria pseudofloribunda M. Fleisch.	Vanuatu, *H. Streimann 62986*	AF509548	AF509943	AF509845
Meteoriopsis squarrosa (Hook.) Broth.	Papua New Guinea, *D. H. Norris 60259*	AF397853	AF417429	AF403666[2]
Meteorium crispifolium Herzog	Papua New Guinea, *B. M. Thiers 3249*	AY457570	AY457571	AY457569
Meteorium papillarioides Nog.	China, *T. Koponen et al. 49966*	AF509551	AF509946	AF509848
Meteorium polytrichum Dozy & Molk.	Australia, *H. Streimann 57477*	AY044073	AF417350	AF395629
Meteorium subpolytrichum (Besch.) Broth.	China, *T. Koponen et al. 55199*	AF509552	AF509947	AF509849
Neodicladiella (*Barbella*) *pendula* (Sull.) W. R. Buck	China, *T. Koponen et al. 53074*	AF397848	AF417417	AF403576
Neodicladiella (*Barbella*) *pendula* (Sull.) W. R. Buck	China, *T. Koponen et al. 50511*	AY044075	AF417426	AF395640
Papillaria crocea (Hampe) A. Jaeger	New Zealand, *H. Streimann 47187* (V. Buchbender)	AF509555	AF509950	AF509852
Papillaria deppei (Müll. Hal.) A. Jaeger	Mexico, *A. Newton 3858* (BM)	AY044078	AF417427	AF395641
Papillaria flavo-limbata (Müll. Hal. & Hamp.) A. Jaeger	New Zealand, *J.-P. Frahm 30-9* (J.-P. Frahm. (CHR)	AF187243 and AF187259	AF397880	AF188048
Papillaria intricata (Mitt.) Müll. Hal. & Broth.	Vanuatu, *H. Streimann 63170*	AF509557	AF509952	AF509854
Papillaria leuconeura (Müll. Hal.) A. Jaeger	New Zealand, 11 Mar. 1994, *W. Frey s.n.* (W. Frey)	AF187244 and AF187260	AF397891	AF188049
Papillaria nigrescens (Hedw.) A. Jaeger	Ecuador, *D. H. Norris 85552, Eduardo Barahona M.*	AY044074	—	AF395632
Papillaria nitens (Hook. f. & Wilson) Sainsbury	Australia, *H. Streimann 64759* (CANB)	AF509556	AF509951	AF509853
Pseudospiridentopsis horrida (Cardót) M. Fleisch.	China, *T. Koponen et al. 55834*	AF397801	AF417383	AF403571
Pseudotrachypus attenuata (Thwaites & Mitt.) W. R. Buck	China, *T. Koponen et al. 57250*	AF397846	AF417438	AF403577
Pseudotrachypus martinicensis (Broth.) W. R. Buck	Dominican Republic, *W. C. Steere 22635* (NY)	AF397841	AF417415	AF403575
Sinskea flammea (Mitt.) W. R. Buck	China, *T. Koponen et al. 54855*	—	AF417441	AF403581
Sinskea phaea (Mitt.) W. R. Buck	China, *T. Koponen et al. 54796*	AF397822	AF417382	AF403572
Toloxis imponderosa (Taylor) W. R. Buck	Equador, *D. H. Norris 90418*	AY044067	AF417391	AF395631
Toloxis semitorta (Müll. Hal.) W. R. Buck	China, *T. Koponen et al. 57247*	AY044079	AF417455	AF395644
Trachycladiella aurea (Mitt.) M. Menzel	China, *T. Koponen et al. 55508*	AF397778	AF417397	AF403573
Trachypodopsis serrulata (P. Beauv.) M. Fleisch.	Taiwan, *I. Ahonen et al. 1511*	AF397849	AF417421	AF403574
Trachypus bicolor Reinw. & Hornsch.	China, *T. Koponen et al. 50721*	AY044060	AF417369	AF395624

Table 1. Continued.

Taxon	Voucher specimen	GenBank accession No.		
		*trn*L-F	*psb*T-H	ITS2
OUTGROUPS				
Amblystegiaceae				
Amblystegium serpens (Hedw.) Schimp.	Finland, *S. Huttunen 1416*	AF397836	AF417420	AF403633
Campyliaceae				
Campylium stellatum (Hedw.) C. E. O. Jensen	Finland, *J. Pykälä 7033*	AF397821	AF417358	AF403609
Hylocomiaceae				
Hylocomium splendens (Hedw.) Schimp.	Finland, *S. Huttunen 1441*	AF397840	AF417408	AF403610
Meteoriella soluta (Mitt.) S. Okamura	China, *T. Koponen et al. 49610*	AF397808	AF417389	AF403606
Rhytidiadelphus triquetrus (Hedw.) Warnst.	Finland, *S. Huttunen 1440*	AF397811	AF417425	AF403631
Hypnaceae				
Ctenidium molluscum (Hedw.) Mitt.	Finland, *J. Pykälä 8706 & J. Nurmi*	—	AF417414	AF403632
Hypnum cupressiforme Hedw.	Finland, *S. Huttunen 1438*	AF397812	AF417361	AF403607
Leskeaceae				
Leskea polycarpa Hedw.	Finland, *J. Pykälä 10872*	AF397810	AF417367	AF403604
Leucodontaceae				
Leucodon sciuroides (Hedw.) Schwägr.	Russia, *S. Huttunen & H. Wahlberg 819*	AF397786	AF417398	AF403634
Neckeraceae				
Neckera complanata (Hedw.) Huebener	Germany, *W. Frey I-4956* (W. Frey)	AY050279 and AY050286	AF122284	AY050295
Orthotrichaceae				
Orthotrichum stramineum Brid.	Sterile culture ex. Capesius, OS-Mo1, currently held in Bonn by J.-P. Frahm	AF127813 and AF129579	AF508317	AF144130
Plagiotheciaceae				
Plagiothecium dentilatum (Hedw.) Schimp.	Finland, *S. Huttunen 1398*	AF397845	AF417419	AF403635
Rhizogoniaceae				
Pyrrhobryum latifolium (Bosch. & Sande Lac.) Mitt.	Taiwan, Sep. 1999, *I. Ahonen et al. s.n.*	AY044077	AF417406	AF395643[2]
Unknown familial placement				
Lepyrodontopsis trichophylla (Hedw.) Broth.	Puerto Rico, *W. R. Buck 21448* (NY)	AY044080	—	AF395642
Lindigia debilis (Mitt.) A. Jaeger	Ecuador, *D. H. Norris 90677 & Eduardo Barahona M.*	AF397803	AF417376	AF403617
Rozea subjulacea Besch.	Venezuela, *Dana Griffin & Manuel Lopez PV-904* (NY)	AF397796	AF417366	AF403578
Rozea andrieuxii (Müll. Hal.) Besch.	Dominican Republic, *W. R. Buck 14205* (NY)	AF397839	AF417409	AF403579

[1] Sequenced using only one primer (ITS3).
[2] Instead of primers ITS3 and ITS4, primers 5.8SR (see Ahonen et al., 2003) and LS4-R (Shaw 2000) were used for PCR and sequencing.

Table 2. Tree lengths, ILD metrics, consistency indices (CI), and retention indices (RI) from analyses based on different analyzing methods and data partitions. Data partitions (ITS2, *trn*L-F, and *psb*T-H sequences and morphology) were analyzed, both independently and combined. In direct optimization (POY 1:1:1) gaps are treated as 5th state, while the PAUP/PRAP analyses of manually aligned data gaps are missing data (PAUP/PRAP 1:1:?).

Analyses and Ti/Tv/indel		ITS2	*trnL-F*	*psbT-H*	Morphology	Combined	ILD	CI	RI
POY 1:1:1		2249	657	547	743	4785	0.130	0.47	0.77
POY 1:1:1	14 morphological characters excluded	2249	657	547	531	4523	0.119	0.50	0.78
POY 1:1:1	Only sequence data	2249	657	547	—	3770	0.084	0.58	0.83
PAUP/PRAP 1:1:?	Only sequence data	986	370	375	—	1867	0.073	0.48	0.76

habitats like cliffs and rocks (P^2 in Table 3; black clades in Fig. 4). Hence, the latter group included a slightly wider selection of species, and in the ancestral state reconstructions for CCT the number of branches having an epiphytic state increased, especially at basal nodes of the cladogram. We are aware that this division of species into epiphytes and non-epiphytes is still rather subjective, but the use of these two different definitions allowed some insight in the robustness of our test.

The effect of correlated characters on the phylogeny reconstruction was studied by performing direct optimization analyses for three different combinations of characters: (1) a combined data set (all morphological + molecular characters), (2) a combined data set excluding morphological characters correlating with epiphytic habit, and (3) molecular data alone. The confidence limits for character correlations were defined using sequential Bonferroni correction (Rice, 1989) to avoid the type I error with multiple statistical tests. Fairly strict limits are supported because of the selection of taxa included in the two major clades, the first representing almost exclusively epiphytic species (Meteoriaceae), whereas the second is mainly non-epiphytic (Brachytheciaceae). Thus, the characters frequently present in the Meteoriaceae as a result of a common ancestry and not because of a shared habitat might lead to significant results in the CCT. This seemed possible, for example, in the case of four characters (Table 3, characters 6, 14, 15, and 16), which were only present in the Meteoriaceae. Sequential Bonferroni correction leads to fairly strict limits and increases the risk of a type II error, but at the same time increases the reliability of the observed correlations (minimization of type I error). For the exclusion of characters, we applied the correlations based on the broader epiphyte definition (Table 3, column P^2 characters in bold) and accelerated character transitions due to its more conservative assumptions on character evolution. The broad epiphyte definition was used because we felt this to be biologically more realistic, as niche selection of species from the facultative epiphytic group does not seem to differ from strict epiphytes.

The character support for all epiphyte clades in the cladogram (Fig. 2) was studied in Winclada (Nixon, 1999b) using unambiguous state changes only. This helped to ensure that epiphyte clades found in combined analyses (Fig. 2) are not only supported by morphological characters correlating with epiphytism (see Wiens et al., 2003). In addition, congruence between morphological and molecular data partitions was tested using the Incongruence Length Difference test (ILD; Mickevich & Farris, 1981; Farris et al., 1995) as implemented in Nona (Goloboff, 1994) in combination with Winclada (Nixon, 1999b). Unlike the ILD metric mentioned above, this test aimed at evaluating the degree of incongruence between data partitions in one analysis. It was performed using 200 replicates of two random addition cycles.

THE CONTRIBUTION OF EPIPHYTISM-CORRELATED CHARACTERS TO THE PHYLOGENETIC STRUCTURE

Using an approach modified from Quandt et al. (2003), we assessed the influence of morphological characters correlating with epiphytism by estimating their impact on the overall phylogenetic structure of the tree. Like Källersjö et al. (1999), we understand "phylogenetic structure" as the robustness of a tree topology as inferred from some estimate of average statistical node support. For the purpose of our present evaluation, we abbreviate the phylogenetic structure as R and define it as:

$$R = (t - 2)^{-1} \sum_{N \in M} S(N) \qquad (1)$$

with t = number of terminals, M = {Nodes N with $S(K) > [(n/2) + 1]^{-1}$}, $S(N) = [j - (n/2) + 1]/[(n/2) + 1]$, n = number of bootstrap or jackknife replicates, and j = number of bootstrapped or jack-

knifed data sets that support the existence of a node *K*. Here we used $n = 100$ and performed bootstrapping with PAUP*, based on the morphological matrix alone, and saving only one shortest tree per bootstrap replicate to reduce computation time.

The mean and standard error of the difference between data sets where all "correlating" sites had been excluded (R_1) and data sets where an equal number of unspecific morphological characters had been excluded (R_2) was estimated by resampling m (here: 500) times, each time randomly choosing different morphological characters for the estimate of R_2. Defining d as $R_1 - R_2$ and l as the index of an individual random iteration, we estimated the standard error as follows:

$$\sigma_d = \sqrt{\frac{1}{m-1}\sum_{i=1}^{m}\left[\left(\frac{1}{m}\sum_{i=1}^{m} d_l\right) - d_l\right]^2} \qquad (2)$$

The resulting probability distribution proved close enough to a normal distribution (Kolmogorov-Smirnov test) to allow that characteristics of the latter could be assumed during the calculation of significance levels and confidence intervals.

Results

Phylogenetic analyses

The analyzed data set consisted of 149 terminals, of which 88 belonged to the Brachytheciaceae, 37 to Meteoriaceae, 7 to Lembophyllaceae, and 15 to other pleurocarpous moss families; 2 outgroups were non-pleurocarpous species from order Bryales (Table 1). Almost all of the DNA sequences were produced in connection with this project, but they were also used in other phylogenetic studies dealing with Brachytheciaceae (Ignatov & Huttunen, 2002; Huttunen & Ignatov, 2004), Meteoriaceae (Quandt et al., 2004b), and Lembophyllaceae (Quandt et al., 2004a). Only very few DNA sequences were missing: of total 149 terminals, *psb*T-H sequence was lacking for 5, *trn*L-F for 4, and ITS2 for 7 terminals. All terminals had at least 2 out of 3 sequenced regions. In the case of one species, *Camptochaete arbuscula*, nuclear ITS2 sequence and chloroplastic *psb*T-H and *trn*L-F sequences originating from different specimens (see Table 1) were compiled to represent a single terminal in phylogenetic analyses.

Analyses of independent molecular and morphological data and congruence between data partitions

Morphological characters alone resulted in 20 parsimonious trees of 743 steps (CI 0.10, RI 0.66). The strict consensus tree of these trees was fairly well resolved (24 unresolved nodes of total of 132 possible nodes in the cladogram), although none of the families studied were monophyletic (results not shown). In contrast, direct optimization analysis of sequence data (*psb*T-H, *trn*L-F, and ITS2) revealed one most parsimonious tree (length 3770, CI 0.58, RI 0.83) with well-resolved topology (Fig. 1; 9 unresolved nodes of total of 139 nodes in the cladogram), which strongly resembled that obtained from combined analyses of molecular and morphological data (Fig. 2).

The ILD test (Farris et al., 1995) revealed a significant incongruence between morphological and molecular data partitions in combined direct optimization analyses ($P = 0.005$). Despite this, we decided to combine these data sets as the topological differences between combined analyses and analyses of independent molecular and morphological data could provide important information of the effects of the correlated characters on phylogenetic groupings.

Direct optimization analyses of combined morphological and molecular data

Direct optimization analyses using POY with an equal weighting for indel, transition, and transversion events produced one parsimonious tree of 4785 steps (CI 0.47, RI 0.77), which is shown in Fig. 2. Its topology is very close to that obtained using only molecular data. Meteoriaceae and Brachytheciaceae were found to be monophyletic, while Lembophyllaceae s.l. (also including *Dolichomitriopsis*, *Bestia*, *Isothecium*, and *Neobarbella*, see Quandt et al., 2004a) was resolved as polyphyletic. This was due to the position of *Neobarbella comes* as sister to *Neckera complanata*. The Brachytheciaceae and Meteoriaceae were resolved as sister clades, whereas the Lembophyllaceae were revealed as not closely related to them. Brachytheciaceae and Meteoriaceae also gained high jackknife and Bremer support (Fig. 2). Differing from current family level classification of mosses (Buck & Goffinet, 2000), *Lepyrodontopsis trichophylla*, *Lindigia debilis*, *Ancistrodes genuflexa*, and *Meteoriella soluta* were not resolved as part of the Meteoriaceae.

In the Brachytheciaceae, four subfamilies each formed a monophyletic clade, although the position of some species differed from recent results by Ignatov and Huttunen (2002). These differences included the position of *Bryoandersonia illecebra*, which was sister to the remainder of Brachytheciaceae in our study, while it was otherwise posi-

Table 3. Results from the concentrated changes test (CCT) for detecting the correlated evolution of epiphytism and 64 morphological characters included in the phylogenetic analyses. Number of steps (L), consistency indices (CI), and retention indices (RI) are reported for each character in cladogram based on analyses of all morphological and molecular data (Fig. 2). For CCT binary coding was applied for all characters. Only character states differing from those used phylogenetic analyses (see Appendix 1) are listed. Test was performed using two different definitions for epiphytism: P^1 indicates results based on a strict concept where only those species are accepted as epiphytes almost always found growing epiphytically, and P^2 represents the broader concept including species also found from other above ground habitats like cliffs and rocks. In these two tests assumption of accelerated character transitions (ACCTRAN) was applied, while in column P^3 results based on delayed character transitions (DELTRAN) together with broad definition of epiphytism. Significant P values after applying sequential Bonferroni correction and characters excluded (i.e., those obtaining significant P^2 value) are with bold font (see text).

Character	L	CI	RI	P^1	P^2	P^3
1. Pendent growth form (0) absent, (1) present	13	7	72	**0.000**	**0.000**	**0.000**
2. Branch foliage (0) terete, (1) complanate	14	7	50	0.623	0.080	0.617
3. Curved branches	24	4	36	0.231	0.040	0.101
4. *Rhynchostegium* whitish color in older parts of shoot	6	16	16	0.282	0.130	0.607
5. Yellowish orange coloration	6	16	37	**0.003**	0.012	0.011
6. Black color in aging shoots	9	11	68	**0.000**	**0.001**	**0.001**
7. Stem central strand	22	4	48	**0.000**	**0.000**	**0.000**
8. Stem cortex thickness	21	4	35	0.023	0.005	0.005
9. Stipe-like pattern	23	4	45	0.010	0.056	0.078
10. Axillary hair color of upper cells	8	12	65	0.858	0.716	0.726
11. Axillary hair upper cell width	3	33	60	0.237	0.688	0.136
12. Axillary hair basal cell	19	5	67	0.617	0.482	0.098
13. Length of axillary hairs	22	4	66	0.237	0.241	0.110
14. Axillary hairs branching or angled	5	20	66	0.026	0.067	0.030
15. Shape of apical cells in axillary hair	3	33	83	0.050	0.091	0.090
16. Flexuous axillary hairs	6	16	28	**0.004**	0.011	0.009
17. Pseudoparaphyllia shape	14	7	35	0.217	0.179	0.228
18. B/M type pseudoparaphyllia	2	50	95	0.261	0.397	0.284
19. Falcate stem leaves	12	8	26	0.994	0.979	0.966
20. Stem leaves appressed (0) absent, (1) present	29	3	47	0.475	0.606	0.474
21. Stem leaves spread almost in 90 degrees angle to shoot (0) absent, (1) present	5	20	33	0.007	0.018	0.013
22. Branch leaves appressed (0) absent, (1) present	28	3	48	0.425	0.536	0.394
23. Branch leaves spread almost in 90 degrees angle to shoot (0) absent, (1) present	6	16	50	**0.004**	0.011	0.009
24. Leaves reflexed	8	12	0	0.384	0.502	0.423
25. Shape of stem leaves (0) acuminate with acumen no less than 1/5th of the leaf length, (1) acute, apiculate or very shortly acuminate	29	3	47	0.312	0.535	0.470
26. Shape of branch leaves (0) acuminate with acumen no less than 1/5th of the leaf length, (1) acute, apiculate or very shortly acumimate	26	3	52	0.250	0.449	0.471
27. Stem leaves piliferous (0) absent, (1) present	5	20	0	0.302	0.437	0.439
28. Leaves with long, flexuous acumen	12	8	35	**0.000**	**0.000**	**0.000**
29. Stem leaf with concave basal part or channeled from the base (0) absent, (1) present	26	3	63	0.435	0.665	0.329
30. Leaf concavity (0) plane, (1) concave all to cochleariform	14	7	35	0.993	0.999	0.999
31. Leaves twisted in mid-leaf when dry	14	7	45	0.287	0.479	0.622
32. Leaves with distinct longitudinal plicae	15	6	48	0.853	0.841	0.837
33. Leaf decurrences	12	8	38	0.797	0.884	0.986
34. Leaves with auricles	8	12	65	0.185	0.167	0.093
35. Leaf margin serrulate to base of leaf	20	5	66	0.311	0.371	0.322
36. Leaf margin of stem leaves (0) serrulate to serrate, (1) slightly serrulate to subentire	27	3	42	0.047	0.025	0.047
37. Costa more than one third of a total leaf width	2	50	50	1.000	1.000	1.000
38. Leaf costa	8	12	50	0.012	0.028	0.029

Table 3. Continued.

Character	L	CI	RI	P^1	P^2	P^3
39. Stem leaf costa with spine	18	5	43	0.981	0.719	0.501
40. Branch leaf costa with spine	23	4	38	0.995	0.826	0.493
41. Costa toothed near its end	11	9	33	0.988	0.959	0.990
42. Leaves recurved at mid-leaf as in *Chrysocladium*	5	20	33	0.071	0.126	0.139
43. Papillose leaf laminal cells (0) absent, (1) present	4	25	91	0.073	0.138	0.132
44. Leaf laminal cells with prorate papillae	5	20	33	1.000	0.969	0.970
45. Alar cells in group lf larger thin-walled cells (0) absent, (1) present	23	4	42	0.995	1.000	1.000
46. Alar cell group of small opaque cells (0) absent, (1) present	28	3	59	0.235	0.331	0.470
47. Alar group shape	13	7	36	0.898	0.868	0.917
48. Basal cells in opaque group across the base	5	20	71	1.000	1.000	1.000
49. Sexual condition (0) autoicous, (1) dioicous (including rarely autoicous dioicous and phyllodioicous species, partly synoicous coded polymorphic)	19	5	52	0.982	0.992	0.997
50. Strioles between high dorsal trabeculae in OPL	13	7	57	**0.002**	**0.000**	**0.000**
51. **Structure of endostome segments** (0) broadly and regularly perforated, (1) narrowly to not perforated, absent or adherent to teeth	15	6	72	0.022	**0.001**	0.005
52. Height of endostome basal membrane	17	5	61	**0.000**	**0.000**	**0.000**
53. **Height of endostome cilia** (0) >0.8 of length of free part of segment, (1) <0.8 or lacking	16	6	73	0.058	**0.002**	0.004
54. Exostome hygroscopic movements	16	6	67	0.010	**0.000**	**0.001**
55. Red color at lower part of exostome	17	5	64	0.010	0.004	**0.003**
56. Width of exostome transition zone	19	5	60	0.008	**0.004**	**0.000**
57. Spores size	22	4	12	**0.000**	**0.000**	**0.000**
58. Reflexed perichatial leaves	18	5	68	0.045	0.020	0.011
59. Height of operculum beak (0) twice or more than height of the rest of operculum, (1) conic	15	6	65	0.937	0.570	0.559
60. Seta surface	23	4	55	0.005	0.007	**0.002**
61. Stomata pore shape	7	14	25	0.071	0.016	0.139
62. Hairy calpytra	11	9	47	**0.001**	**0.002**	**0.003**
63. Seta length (0) longer than branch length, (1) shorter than branch length	17	5	54	**0.000**	**0.000**	**0.000**
64. Capsule orientation	18	5	69	0.053	**0.002**	**0.000**

tioned as member of Rhynchostegielloideae (Ignatov & Huttunen, 2002). Moreover, *Myuroclada maximowiczii* was in our direct optimization analysis sister to the Rhynchostegioideae–Homalothecioideae–Brachythecioideae clade and not within the Brachythecioideae as in Ignatov and Huttunen's (2002) classification, as well as *Scleropodium obtusifolium* and *Kindbergia praelonga* are here within the Homalothecioideae clade instead of grouping within the Brachythecioideae.

The Meteoriaceae clade included two lineages. One consisted of members of *Cryptopapillaria*, *Meteorium*, *Papillaria*, and *Toloxis*, as well as *Chrysocladium retrorsum*, *Diaphanodon blandus*, and *Trachypus bicolor* (Fig. 2). Within this clade two well-supported groups were consistent, (1) the *Papillaria* clade with all *Papillaria* species except *P. nigrescens* and *P. deppei*, plus *Cryptopapillaria* and *Toloxis*; and (2) the *Meteorium* clade with *Chrysocladium retrorsum*, *P. nigrescens*, *P. deppei*, and all *Meteorium* species. *Diaphanodon blandus* was resolved sister to the *Meteorium* group. The second lineage in the Meteoriaceae (A clade in Fig. 2) was morphologically more variable and included the former Trachypodaceae such as *Trachypodopsis serrulata*, *Duthiella wallichii*, and *Pseudospiridentopsis horrida*, and more "traditional" members of the Meteoriaceae, such as *Aerobryidium*, *Aerobryopsis*, *Meteoriopsis*, *Barbella*, *Neodicladiella*, *Barbellopsis*, *Trachycladiella*, *Pseudotrachypus*, *Sinskea*, and *Floribundaria*.

Direct optimization resolved the Hylocomiaceae (with three species, *Hylocomium splendens*, *Rhytidiadelphus triquetrus*, and *Meteoriella soluta*) as closest relative of the Meteoriaceae–Brachytheciaceae clade. The Lembophyllaceae were not close relatives of the Meteoriaceae and Brachytheciaceae, but within the same clade as *Leskea poly-*

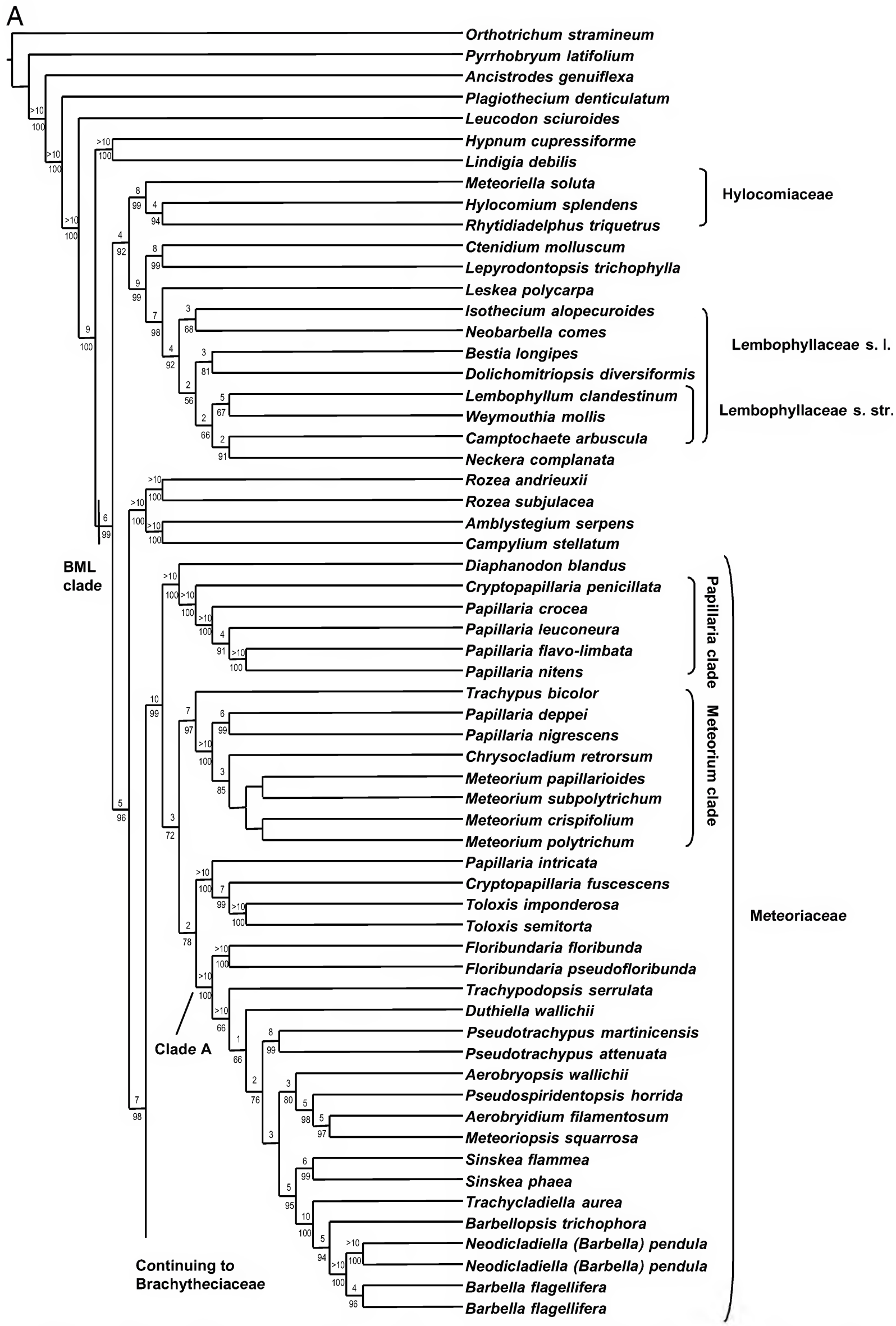
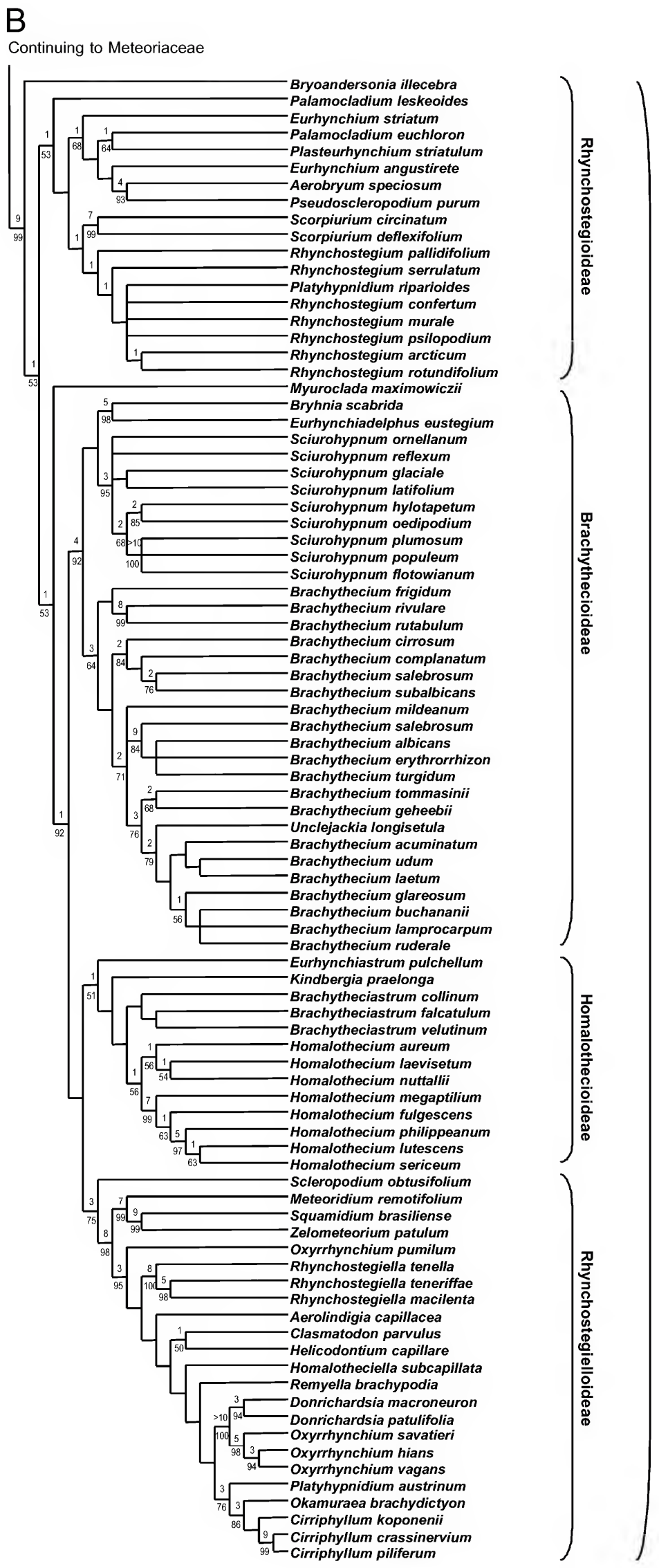


Figure 1. The only parsimonious tree (length 3770, CI 0.58, RI 0.82) based on direct optimization analyses of molecular data (cp *psb*T-H and *trn*L-F, and nr ITS2). Jackknife support for clades is indicated below the branches and Bremer support above these.



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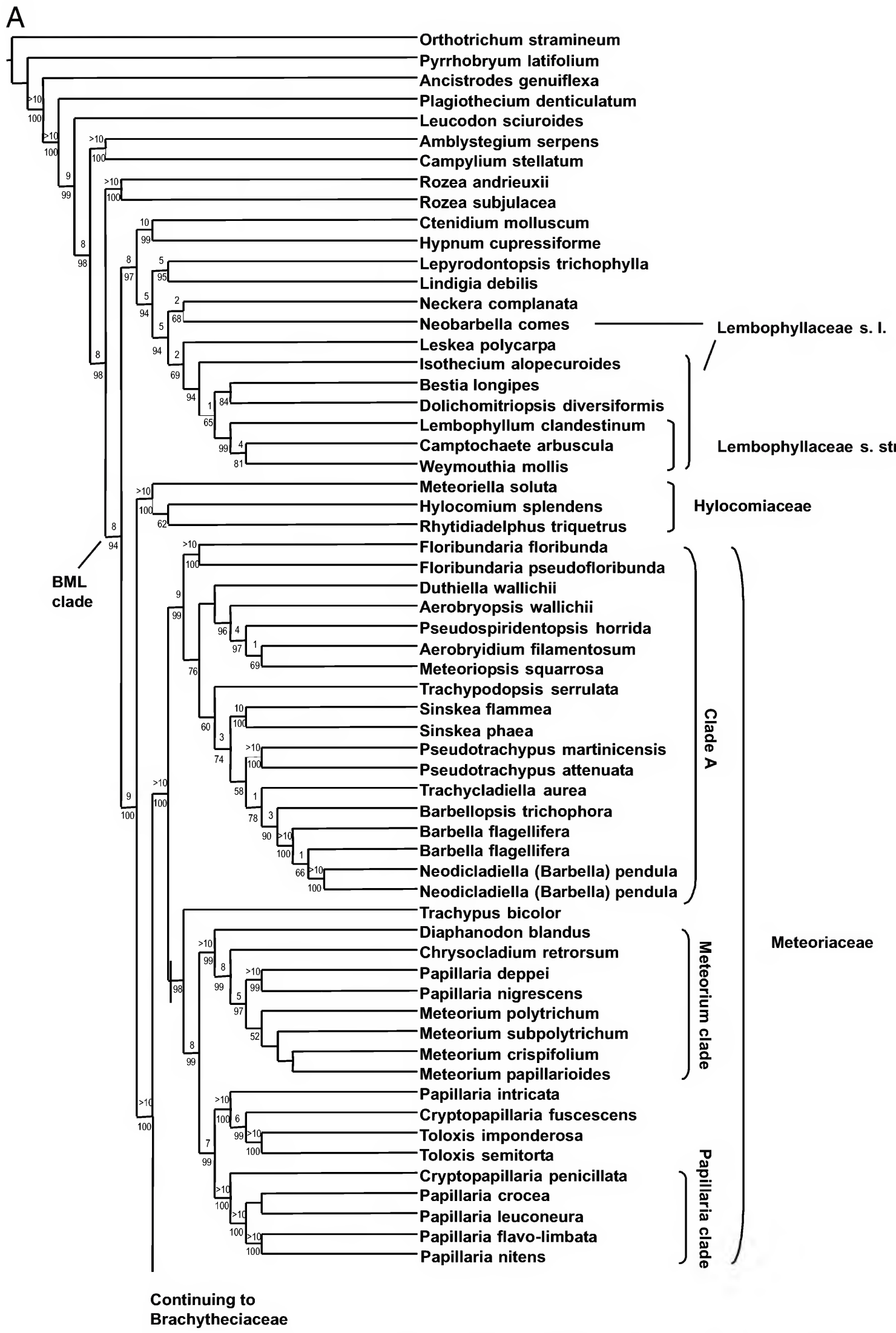

Figure 2. The only most parsimonious (length 4781, CI 0.48, RI 0.76) tree based on direct optimization analyses of combined morphological and molecular data. Jackknife support for clades is indicated below the branches and Bremer support above these.

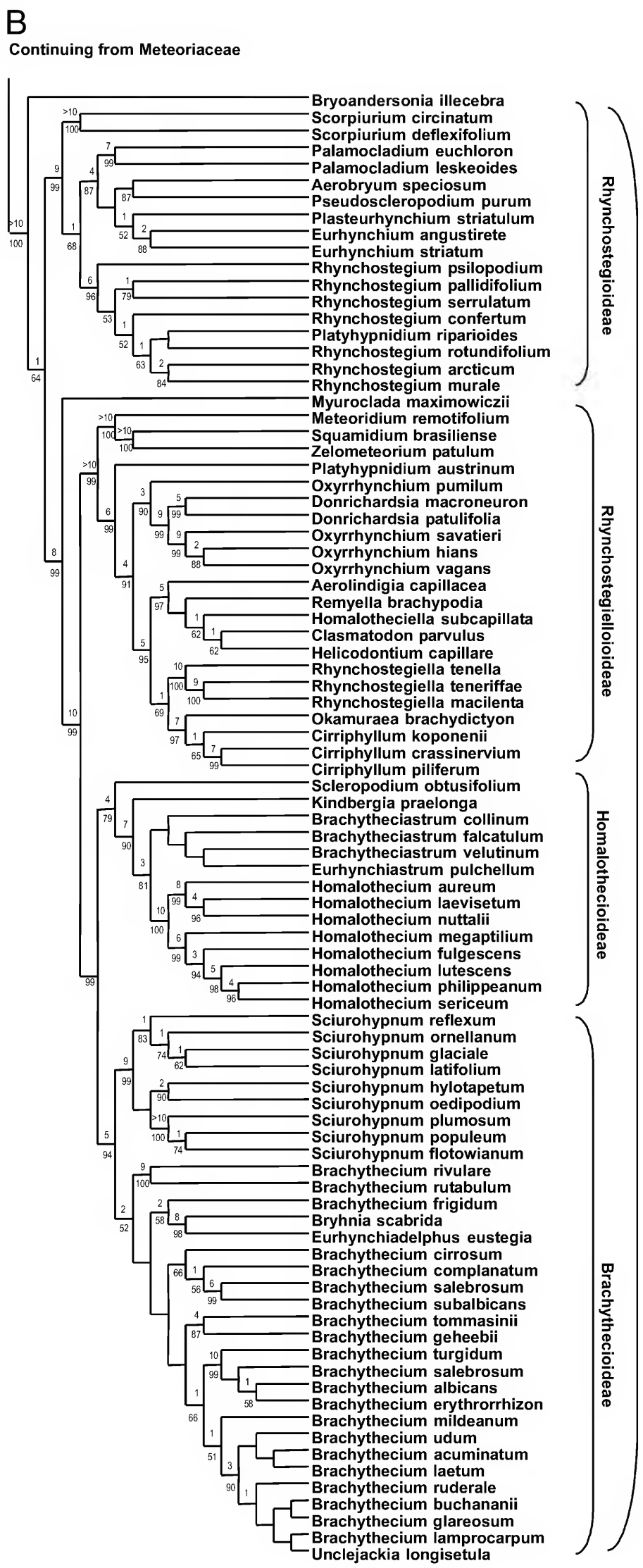

Figure 2. Continued.

carpa, the Hypnaceae (*Ctenidium molluscum* and *Hypnum cupressiforme*), *Lindigia debilis*, and *Lepyrodontopsis trichophylla*.

A major difference between the topology based on only molecular data (Fig. 1) and the combined analyses of morphological and molecular data (Fig. 2) was the branching order of clades within the Meteoriaceae and the position of Hylocomiaceae. The direct optimization of molecular data did not resolve the *Meteorium/Papillaria* clade as monophyletic. The members of the *Papillaria* clade were resolved together with *Diaphanodon blandus* in a position basal to other Meteoriaceae, whereas *Meteorium* species along with *P. nigrescens*, *P. deppei*, *Chrysocladium*, and *Trachypus bicolor* form a monophyletic group sister to clade A. While combined analyses resolved Hylocomiaceae as sister to the Brachytheciaceae–Meteoriaceae clade, molecular data alone supported their position as basal to the clade including the Lembophyllaceae.

Parsimony ratchet analyses of manually aligned molecular data

The topology of the strict consensus tree of 1054 parsimonious trees (length 1867, CI 0.48, RI 0.76) found during analysis of the manually aligned data set (Fig. 3) also resolved the Meteoriaceae and Brachytheciaceae as sister groups. As in direct optimization analysis of the molecular data (Fig. 1), all four subfamilies of the Brachytheciaceae were monophyletic, and their circumscriptions are in conflict with Ignatov and Huttunen's (2002) findings only in the position of *Scleropodium obtusifolium* (basal to Homalothecioideae–Brachythecioideae clade instead of belonging to Brachythecioideae), and *Myuroclada maximowiczii* (basal to the Rhynchostegielloideae–Homalothecioideae–Brachythecioideae clade instead of belonging to the Brachythecioideae). Out of the four subfamilies, the Rhynchostegioideae and Homalothecioideae received weak jackknife or Bremer support, whereas the Rhynchostegielloideae and Brachythecioideae received jackknife values above 80% (Fig. 3).

Topologies from direct optimization analyses and analyses of manually aligned data were congruent on the subfamily and genus level. Most of the differences occurred in weakly supported clades. For example, different analysis methods placed three species, *Bryoandersonia illecebra*, *Kindbergia praelonga*, and *Scleropodium obtusifolia*, in different subfamilies (Figs. 1, 3). Unlike the analyses of manually aligned data, direct optimization did not resolve *Palamocladium* as monophyletic.

Unlike in direct optimization analysis (Figs. 1, 2), a clade consisting of *Trachypus bicolor*, *Toloxis*, *Papillaria intricata*, and *Cryptopapillaria fuscescens* was found to diverge first in the Meteoriaceae (Fig. 3). In the topology obtained from POY analyses of only molecular data (Fig. 1), the clade including *Cryptopapillaria* and *Toloxis* species was within clade A, and the *Papillaria* clade also including *Diaphanodon blandus* was basal to Meteoriaceae clade. The *Meteorium/Papillaria* clade consisted in PAUP/PRAP analyses of a *Meteorium* clade including all *Meteorium* species and a *Papillaria* clade with *Chrysocladium*, *P. nigrescens*, and *P. deppei* in one clade, sister to a clade comprising *Cryptopapillaria penicillata* together with the remaining *Papillaria* species (Fig. 3). Most of the species that were resolved as part of clade A (in direct optimization; Figs. 1, 2) were found to constitute one clade in the PAUP/PRAP strict consensus tree as well (clade A in Fig. 3). This clade included three groups with unresolved relationships: one with *Sinskea*, *Trachycladiella*, *Barbellopsis*, *Neodicladiella*, and *Barbella*; another including *Meteoriopsis*, *Aerobryidium*, *Aerobryopsis*, *Pseudotrachypus*, and *Duthiella*; and a third with two *Floribundaria* species, *F. floribunda* and *F. pseudofloribunda*. *Trachypodopsis serrulata* occupied an unresolved position in clade A.

As in the direct optimization analysis of all data (Fig. 2), the Hylocomiaceae were closest relatives of the Meteoriaceae and Brachytheciaceae, although the position of *Ctenidium molluscum* as sister to *Hylocomium* left the Hylocomiaceae as a nonmonophyletic entity. In the POY analyses of molecular data only (Fig. 1), the clade composed of the Amblystegiaceae and *Rozea* was resolved as sister to the Meteoriaceae–Brachytheciaceae clade. PAUP/PRAP analyses of manual alignment (Fig. 3) did not resolve the Lembophyllaceae as monophyletic, but as in the direct optimization analyses of molecular data (Fig. 2), *Neckera complanata* was sister to *Camptochaete arbuscula* within the Lembophyllaceae s. str. (sensu Tagney, 1997). This placement, however, could be an artifact resulting from lacking ITS2 data. Without this placement of *Neckera*, the Lembophyllaceae s.l. (Quandt et al., 2004a) would be monophyletic. In the analyses of manual alignments, all clades received generally much lower support and generic relationships remained unresolved in some clades (*Rhynchostegium*, *Brachythecium*, *Sciurohypnum*).

EVOLUTION OF EPIPHYTISM AND EPIPHYTIC CHARACTERS

In the light of our phylogenetic analyses, and based on the conservative assumption of acceler-

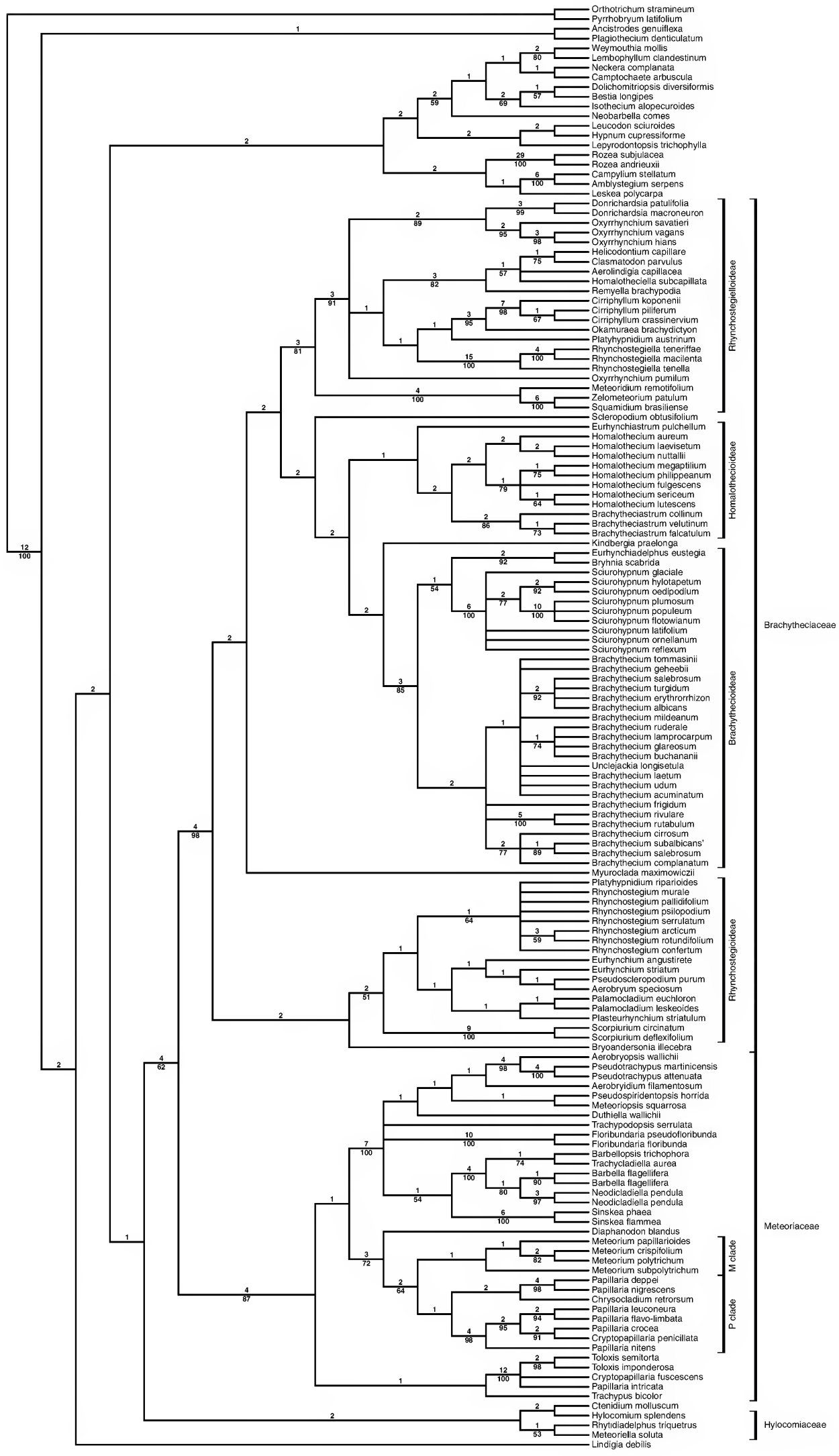

Figure 3. Strict consensus tree of 1054 parsimonious trees (length 1867, CI 0.45, RI 0.76) found during the parsimony ratchet of the molecular data. Jackknife support is indicated below the branches, while the Bremer support is indicated above.

ated transformations, the utilization of epiphytic habitats appears to have happened at least 10 times independently within the clade including members of the Brachytheciaceae, Meteoriaceae, and Lembophyllaceae (BML clade, Fig. 4), depending on how strictly epiphytism was defined. With strict confidence limits, 14 of all morphological characters were significantly correlated with the evolution of epiphytism if broadly defined (P^2, Table 3), and 12 if a narrow definition was applied (P^1, Table 3). When delayed character state transitions were expected together with broad definition of epiphytism, CCT supported the correlated evolution of 14 characters, which were however slightly different from those obtained with assumption of accelerated transitions (P^3, Table 3). All approaches supported the correlated evolution of 9 characters, 5 of which were sporophytic and 4 gametophytic. The character states of the 14 characters supported by accelerated character state transitions are given in Figure 4. Character states that are often stated to be adaptations to epiphytism (see Discussion) are concentrated within clades with mainly epiphytic species (Fig. 4). This is especially striking within the Brachytheciaceae, where epiphytism has evolved independently at least once in each subfamily, and where character states connected to epiphytism are almost completely restricted to those epiphytic lineages.

CONTRIBUTION OF CORRELATED EVOLUTION OF EPIPHYTE CHARACTERS TO PHYLOGENETIC STRUCTURE AND INFERRED HYPOTHESES OF RELATIONSHIPS

When the 14 characters correlated with epiphytism were excluded from analyses, direct optimization analyses resulted in one most parsimonious tree of 4523 steps. This cladogram is thus 262 steps shorter than that obtained from analyses where all morphological characters are included (Fig. 2). The topology of this tree was well resolved. The exclusion of correlated characters decreased the incongruence between data partitions (Table 2) and, according to the retention and consistency indices, decreased the homoplasy (including 14 correlated characters CI 0.47 and RI 0.77, when these were excluded CI 0.50, RI 0.78). This decreasing homoplasy should not be surprising due to the observed incongruence between molecular and morphological data. However, the excluded morphological characters are not significantly more homoplasious than other morphological characters (Table 3), although most of them show frequent transitions between character states in the most parsimonious cladogram. Grouping of species was almost identical though some changes appeared mainly among outgroup species. However, the exclusion did not affect grouping of epiphyte species as all epiphyte clades within the BML clade were similar. The only major difference in the grouping of epiphytes was the Lembophyllaceae s.l. clade, which after exclusion had the same topology as observed in the tree based on molecular data (Fig. 1).

An examination of the characters supporting clades in the cladogram obtained by direct optimization (Fig. 2) revealed that all the epiphyte clades were consistently supported by both molecular and morphological characters, and none of them was supported only by characters correlated with epiphytism. This suggests that the presence of these clades is not depending only on characters correlated with epiphytism. Those clades with the lowest number of supporting characters were surprisingly found within *Homalothecium*, clades consisting of *H. laeviseta* and *H. nuttalii* (four substitution events in the ITS2 region and one morphological character (50), which is correlated with epiphytism), and *H. fulgescens*, *H. lutescens*, *H. philippeanum*, and *H. sericeum* (one substitution event in the *psb*T-H region and one morphological character (55) correlated with epiphytism). In general, the *Homalothecium* clade and clades within it were, however, among the most stable clades within the Brachytheciaceae.

The exclusion of correlated characters positively affected the phylogenetic structure (R). R was significantly higher when the characters correlated with epiphytism were excluded than when an equal amount of any morphological characters is randomly selected and deleted. Reflecting the low number of morphological characters that were used to resolve the relationships among a comparably high number of terminals, the overall R indices were low. This, however, applies equally to R_1 and R_2, and only the difference (d) between both was assessed by our test. d was calculated as 0.0140 with a standard error of 0.0058. In a two-tailed Z-test, equality of R (H_0: $d = 0$) could be rejected at $0.05 > P > 0.01$. The upper and lower boundaries of a 95% confidence interval were 0.0027 and 0.0253, respectively.

Discussion

PHYLOGENY RECONSTRUCTION AND EFFECT OF ALIGNMENT METHOD ON OBTAINED TOPOLOGY

The inferred hypotheses on phylogenetic relationships among Brachytheciaceae, Lembophyllaceae, and Meteoriaceae were similar irrespective of

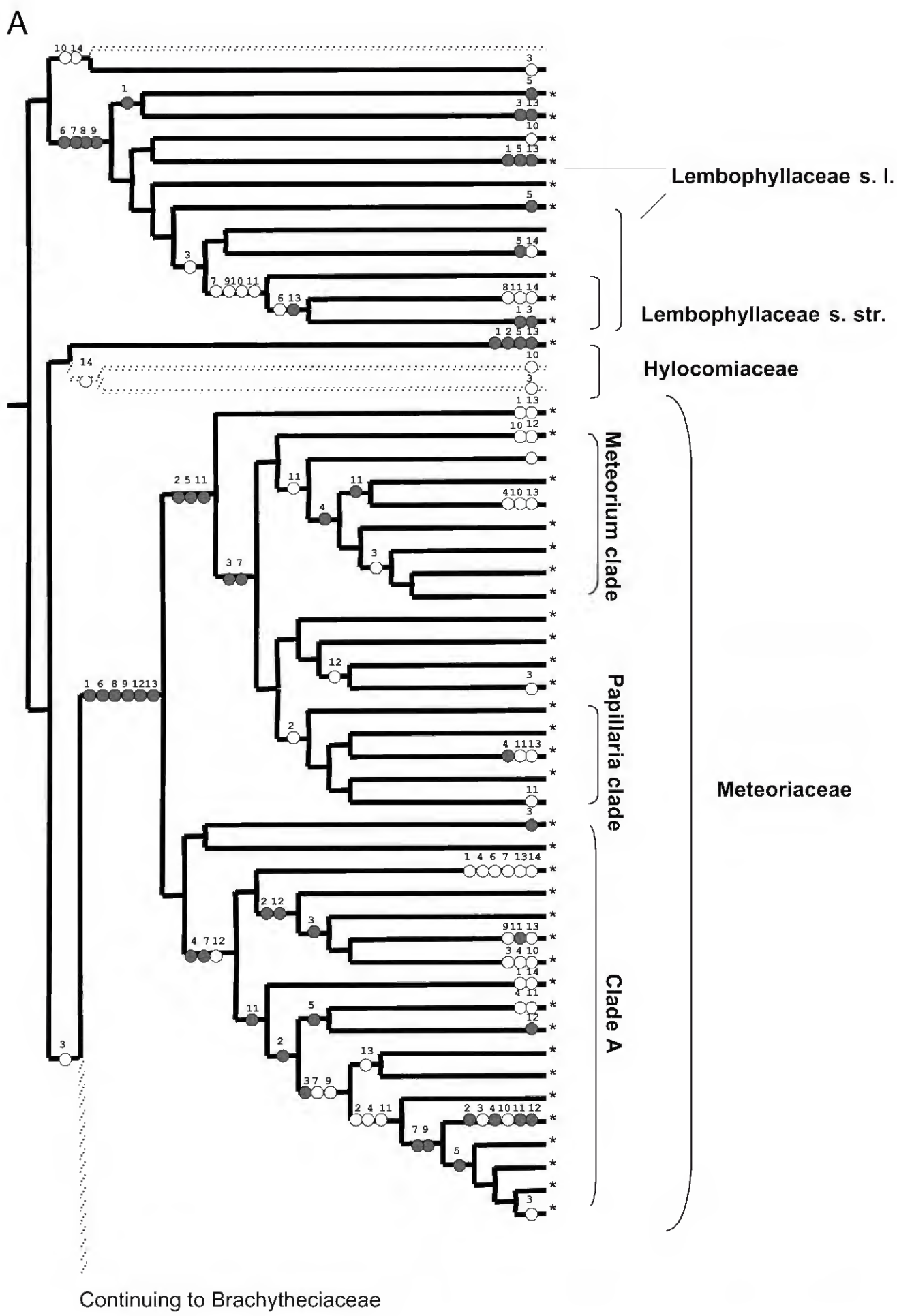

Figure 4. The evolution of epiphytism (black branches) and character states of the characters correlating with epiphytism in clades including the Brachytheciaceae, Meteoriaceae, and Lembophyllaceae. Character state transitions are marked along branches, numbers above clades indicate numbers of characters (see Table 3). Gray circle indicates transitions from character state 0 to 1 and open circle from 1 to 0. The characters are optimized using ACCTRAN optimization. Species that are also growing on cliffs and stones but at least sometimes found as epiphytes are regarded as epiphytes (epiphytes s.l.; see Methods for further information). The species almost exclusively found as epiphytes (epiphytes s. str.) are marked with asterisks.

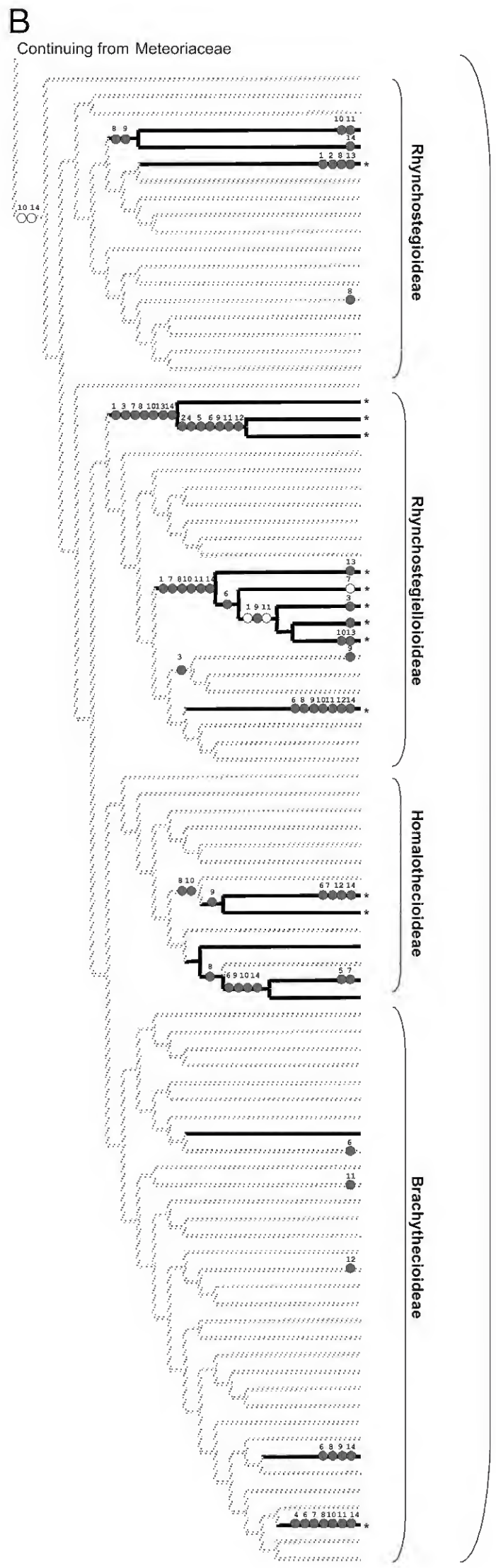

Figure 4. Continued.

the analytical method used. This is encouraging when taking in consideration that in addition to differences in homology statements between manual sequence alignment and POY implied alignments, these methods also differed in the treatment of gaps. Information from insertion and deletions are used in POY for phylogeny reconstruction in the same way as all other characters (i.e., as fifth character state), while in PAUP/PRAP gaps were treated as missing data. This impacted the numbers of parsimony informative (pi) positions and amount of available information for phylogeny reconstruction. If the single character optimization obtained from POY analyses of all available data is studied as a static alignment, 870 out of the total of 2365 DNA characters were informative when gaps were treated as fifth character state (*trn*L-F, 640 bp, 186 pi positions; *psb*T-H, 582 bp, 126 pi; ITS2, 1143 bp, 558 pi). In the same character optimization only 383 pi characters (*trn*L-F, 95 pi positions; *psb*T-H, 93 pi; ITS2, 195 pi) were available if information from indel events was ignored. As observed earlier (Quandt et al., 2004b), this is probably the reason for lower support and resolution of strict consensus topologies based on the manual alignment (Figs. 1, 3). Based on results of this study as well as some earlier comparisons between direct optimization and other phylogenetic methods (Wheeler & Hayashi, 1998; Quandt et al., 2004b), it seems that direct optimization as implemented in the program POY is able to handle even very variable DNA sequence data like ITS2 in our study. Manual alignments are often criticized as subjective (Giribet et al., 2002), because hypotheses of positional homology in non-coding DNA sequence regions may be dependent on experience and personal preferences. In the present absence of a thorough understanding of all microstructural changes that underlie alignment gaps (Graham et al., 2000; Kelchner, 2000) and a general indel coding scheme inferred from such knowledge (see Simmons & Ochoterena, 2000, for a critique of the 5th-character-state approach of POY and a discussion on alternatives), POY at least guarantees objectivity and in our study does not deviate significantly in the inferred hypotheses from the standard approach.

PHYLOGENETIC RELATIONSHIPS AND TAXONOMIC IMPLICATIONS

Like most earlier studies including members of the Meteoriaceae and Brachytheciaceae (Huttunen & Ignatov, 2004; Quandt & Huttunen, 2004; Quandt et al., 2004b), all results in this study support the close relationship and monophyly of these families. In analyses including the morphological data, the Hylocomiaceae were resolved as closest relatives of the Meteoriaceae–Brachytheciaceae clade (Fig. 2). Although there are only very few studies that include a representative number of species from these families, at least some of them resolve the Meteoriaceae and the Brachytheciaceae as close relatives, but almost never as sister groups (Buck et al., 2000a, b; DeLuna et al., 2000; Arikawa & Higuchi, 2003). The analyses by Buck et al. (2000b) resolved the Plagiotheciaceae as closest relatives to the Brachytheciaceae, but despite this the authors already suspected a connection between Meteoriaceae and Brachytheciaceae probably due to morphological similarities (see Buck, 1994).

Our morphological studies of pseudoparaphyllia, which cover the young branch primordia, revealed some misinterpretations in earlier studies (Ignatov, 1999; Ignatov & Huttunen, 2002; Huttunen & Ignatov, 2004). Thus, the value of this character in delimiting the Meteoriaceae and Brachytheciaceae appears more reliable than expected. Closer observations of the structure of branch primordia in *Neobarbella comes* and *Lindigia debilis* showed that they differ from the Meteoriaceae–Brachytheciaceae type of pseudoparaphyllia. In fresh specimens of *L. debilis* we observed filamentous pseudoparaphyllia surrounding branch primordia, which are absent from species in the Meteoriaceae–Brachytheciaceae clades. *Neobarbella comes* revealed some variation in branch primordial structure even within a single shoot, but in most of the cases additional ridges or narrow leaf-like pseudoparaphyllia are present obliquely between adjacent leaf and young branch primordia. As suggested by Ignatov (1999), the specific type of branch primordial pattern appears to be a synapomorphy for a Meteoriaceae–Brachytheciaceae clade, although this character state was probably lost in one species, *Trachypus bicolor*.

Although our selection of the Lembophyllaceae species is rather limited, including only 7 genera of a total of 14 placed in the family (sensu Quandt et al., 2004a), our results are consistent with those obtained in more detailed studies based on molecular data (Quandt et al., 2004a). The Lembophyllaceae are closely related to the Neckeraceae, which is not a novel finding as Brotherus (1925) and Robinson (1975) previously considered them as closely related.

EVOLUTION OF CHARACTERS CORRELATING WITH EPIPHYTISM

Our analyses confirmed the correlated evolution of epiphytism and several morphological characters

within Brachytheciaceae, Meteoriaceae, and Lembophyllaceae (see Table 3). Several earlier authors (Grout, 1908; Patterson, 1953; Vitt, 1981; Buck & Vitt, 1986; Buck, 1991; Hedenäs, 1998; Newton & De Luna, 1999) described some aspects of this. Both Hedenäs (2001) and Vanderpoorten et al. (2002) were able to demonstrate the correlation between epiphytism and some morphological characters. Vanderpoorten et al. (2002) made a similar observation in the Amblystegiaceae, providing evidence that at least the evolution of upright capsules, low endostome basal membrane, and length of endostome cilia are correlated with a transition to epiphytism. Hedenäs's morphological study (2001) of 439 pleurocarpous moss species revealed that the lack of a well-developed central strand in the stem as well as the length of the seta, a papillose or smooth lower exostome outer surface, and large spores are characteristic for epiphytic species, which is corroborated by the present study. Although most of the correlated characters revealed here are the same as those suggested previously, our study also revealed that a blackish coloration in the aging shoot, and a long, flexuous leaf acumen are most likely associated with epiphytic habitats. Both of these characters are common in the Meteoriaceae, but evolved independently outside this family. In early classifications all species with these two character states (e.g., *Aerobyum speciosum, Meteoriella soluta, Squamidium brasiliense, Zelometeorium patulum*) were placed in Meteoriaceae (Brotherus, 1925; Vitt, 1984); hence, these characters were regarded as "typical Meteoriaceae features" rather than resulting from convergent evolution to epiphytic habitats. Instead of simply being advantageous in any epiphytic habitats, the evolution of blackish coloration and pendent growth, for example, might well be connected to tropical and subtropical habitats. Both of these character states are only present in those epiphytes which mainly occur in humid tropical or temperate areas.

In addition to differences due to selected study group, assumptions applied in CCT had a minor effect on detected correlations. Of those three alternatives studied here (Table 3, P^1, P^2, and P^3), all supported the correlated evolution of the same nine characters. Thus, this set of characters probably provides the most promising starting point for the further studies on adaptive evolution.

Epiphytism has led to parallel evolution of reduced peristome structures in all Brachytheciaceae subfamilies. Within Meteoriaceae, as well as in *Aerolindigia, Squamidium, Homalothecium,* and *Aerobryum,* most species have peristomes enabling the spore dispersal in wet conditions, large spores, endostomes with a low basal membrane, narrowly to imperforate segments, and reduced or absent cilia. The transversal striae at the base of the outer surface of the exostome teeth are often lacking, and because of an expanded transition zone and minor deposition of material on inner surface of exostome teeth (see Ignatov et al., 1998), the hygroscopic movements of the exostome teeth are weak. The prevalence of these character states concentrates on those clades comprising epiphytic species (Fig. 4).

The arguments for the adaptive nature of correlated characters are mostly logically explained. For example, the xerocastique hypnoid peristome that is releasing spores under dry conditions is most common in terricolous species with a double peristome. In rain and fog the peristome closes the mouth of capsule, preventing the release of spores when conditions are unfavorable for wind dispersal. The peristome then opens during dry periods, with the most vigorous movements to release the spores occurring during initial dryness. In contrast to this, hygrocastique peristomes, usually with relatively weak hygroscopic movements, are open when wet, allowing spores to be washed out by rain drops and flow down the trunk, reaching bark cracks, etc. (Lazarenko, 1957; Neumann & Mueller, 1987). In addition to epiphytes, this peristome type is frequently found also in obligate epilithic mosses (e.g., *Schistidium, Seligeria,* etc.). Spores of these species have a chance to remain on the surface, either trunk or rock, and germinate only as long as it is wet and sticky for spores. In these species larger spores may be selectively beneficial, allowing the faster germination. The increase in the spore size has been speculated to be one reason for the higher number of local endemics in epiphytes (and epilithic species) than in terricolous species (Iwatsuki, 1972).

Unlike Vanderpoorten et al. (2002) and Hedenäs (2001), we did not find lid shape, sexual condition, shape of stomata pore, and presence of decurrent leaves to correlate with the evolution of epiphytism. This may be due to our selection of species, which was probably not the most advantageous for detecting correlated evolution of epiphytic characters. The inclusion of one large clade comprising exclusively epiphytic species (Meteoriaceae) could lead to difficulties in separating the correlations resulting from environmental conditions or shared phylogenetic history. The correlations resulting from close relatedness could be eliminated, for example, by selecting pairs or smaller groups of epiphyte and non-epiphyte species from several independent moss lineages and investigating the correlations among this group.

Despite the possible adaptive evolution, for example, the degree of reduction and combinations of sporophytic characters often show a fairly wide variation even between very closely related epiphyte species. Within those families studied here, the sporophyte characters in epiphytic Brachytheciaceae seem to be in general less modified or specialized than in the Meteoriaceae. Only very few Brachytheciaceae species lack the cross striate ornamentation at the base of the exostome. The exostome transition zone, where ornamentation on the outer surface often changes from cross-striate to papillose and the exostome teeth narrow more abruptly forming a shoulder-like outline, is only rarely more than four plates. In our study, only four Brachytheciaceae species with these characters are included, *Homalothecium philippeanum*, *Clasmatodon parvulus*, *Zelometeorium patulum*, and *Squamidium brasiliense*. Less modified peristomes in the Brachytheciaceae might be the result of a "phylogenetic time lag" (Harvey & Pagel, 1991: 41). This means that the current phenotype of the organism might still partly reflect the past ecological conditions, as all epiphyte lineages in Brachytheciaceae have evolved from an ancestor with a perfect hypnoid peristome type. Unlike in the Meteoriaceae and the Lembophyllaceae, a very clear tendency towards more reduced peristomes is observed in most Brachytheciaceae lineages that comprise epiphytic species. In the Meteoriaceae, morphological variation of sporophytic characters is confusingly homoplastic especially in clade A (Fig. 3), while in the clade including all *Meteorium* and *Papillaria* species the peristome structure in all species is rather conserved.

Sporophytic characters have historically been considered highly conserved and therefore interpreted as reliable indicators of higher level relationships. Like several other recent studies, our investigation shows them to be at least as sensitive to environmental conditions as many gametophytic characters (Hedenäs, 1999; Buck et al., 2000a; Hedenäs, 2001; Vanderpoorten et al., 2002). Based on our results, it seems that instead of being stable and independent of ecological conditions, sporophytic characters are more sensitive to environmental changes than those of the gametophyte generation. Hedenäs (1999), Buck et al. (2000b), and Vanderpoorten et al. (2002) came to the same conclusion when examining the evolution of morphological characters. In our analyses, 10 out of 15 sporophytic characters are correlated with a shift to epiphytic habitat compared to only 4 out of a total of 49 gametophytic ones.

CONTRIBUTION OF EPIPHYTE CHARACTERS TO PHYLOGENY RECONSTRUCTION

Although the species growing in epiphytic habitats share several morphological characters, the inclusion of these characters in phylogenetic analyses does not significantly compromise the phylogenetic signal originating from other morphological and molecular characters. Both analyses of sequence data alone (Fig. 1) and the combined molecular and morphological data (Fig. 2) resolved the Meteoriaceae and Brachytheciaceae as monophyletic and pointed toward several independent origins of epiphytism. In some studies, including the characters that are correlated with specific ecological condition has led to topologies where unrelated species with similar habitat preferences form clades (e.g., McCracken et al., 1999; Wiens et al., 2003). This is one reason why the inclusion of characters correlated with environmental conditions has often been stated to lead to biased conclusions of phylogenetic relationships (Emerson & Hastings, 1998; Quicke & Belshaw, 1999; Wiens et al., 2003). The opposite findings, however, also exist to show convergent characters as improving the structure in the cladogram (Branham & Wenzel, 2003). We found that in the topologies based on (1) combined analyses of all data, (2) analyses excluding correlated characters, or (3) including only molecular data, the relationships between epiphytic and non-epiphytic taxa were congruent. Thus, biased results appear not to apply in our case (see De Queiroz, 1996). Wiens et al. (2003) listed three criteria for detecting whether the phylogenetic analyses may be affected by convergence. First, a clade including species with a similar selective environment should have strong morphological support. Second, the characters supporting this clade should consist of only characters correlating with the shared selective environment, and third, there is phylogenetic evidence that those species are not actually a monophyletic group. Our results do not point toward any of these criteria. Hence, it seems that in our study the inclusion of correlated characters in phylogenetic analyses does not entail those problems frequently associated with the use of convergent or parallel characters. Some negative effects, however, did follow from their inclusion. We expect that at least part of the incongruence between the molecular and morphological data is result of correlated evolution of morphological characters in epiphytic species. Correlated characters also increased incongruence between data partitions and homoplasy and negatively affected the robustness of the obtained phylogenetic hypothesis.

Buck (1994) suggested that at least some features, like hairy calyptras in epiphytic species, could be just by-products of evolution of some other characters with a real adaptive value. This idea would indicate that not all characters linked to epiphytism are evolving independently. This was confirmed in study by Hedenäs (2002), which showed several morphological characters to correlate with each other. The non-independent characters, however, pose some methodological problems in phylogenetic analyses. In phylogenetic analyses one of the basic assumptions is that all characters included in study are independent and hence that each provides a unique evidence for common ancestry (Kluge, 1989). If some of them appear not to evolve independently, but instead are linked to each other, inclusion of both these characters will lead to extra weighting of one evolutionary event and possibly change the obtained hypotheses of relationships. Compared to sequence level data, the independence of morphological characters is often difficult to demonstrate. Although the correlation of two character transformations may indicate that their evolution is linked together, even this does not confirm the non-independency of them (Buckup, 1993; O'Leary & Geisler, 1999). The interactions of morphological characters may be the result of developmental, functional, and genetic interactions (Emerson & Hastings, 1998), and most often there is not enough information on evolution, genetic regulation, function, and development to evaluate this independence of characters.

Ecological constraints affecting morphological characters might also be a reason for the incongruence between the phylogenetic signal from morphology and molecular data. This could be detected by using the partitioned Bremer support method (Baker & DeSalle, 1997; Baker et al., 1998), which assigns the contribution of each data partition separately for each clade. Unfortunately, current software designed for those analyses is unable to give reliable results for data sets as large as ours. As shown by Wahlberg and Nylin (2003), the incongruent data partitions may still positively contribute to the branch support of individual nodes in a cladogram, leaving only very few clades that produce the observed conflict between data partitions.

Although certain morphological modifications in pleurocarpous mosses seem to be a general consequence of epiphytic habitats, the evolution of these modified characters still entails different character combinations. Some marks of independent evolutionary routes may still remain between different groups and appear useful in phylogeny reconstruction (Newton & De Luna, 1999). In reduced peristomes, for example, very striking morphological differences can sometimes be found, despite similar habitats and ecology of epiphytic species. If the modifications are the result of selective pressures, evolutionary potential and the response to selection may differ between species and lead to different character combinations. In the case of epiphytic mosses, habitats show a wide variation in environmental factors, each of which probably plays a slightly different role in different habitats (Hedenäs, 2001). As result of this, even species adapted to epiphytic habitats could potentially differ because of variation in these epiphytic habitats due to, for example, climatic or geographical differences. They may as well result from different evolutionary age in those lineages. This could explain why the parallel or convergent evolution of these characters after all does not seriously impact the phylogenetic analyses.

Conclusions

All methods used in our study revealed a well-resolved phylogeny for the Meteoriaceae, Brachytheciaceae, and Lembophyllaceae. We observed several independent origins of epiphytism within these three families and demonstrated that the evolution of certain morphological characters, such as reduction in several sporophytic characters, correlates with the utilization of epiphytic habitats. Bryologists have for a long time noted the tendency to modified sporophyte structure and reduced peristomes in epiphytic mosses (for example, Grout, 1908; Patterson, 1953; Hedenäs, 2001). Probably also due to the apomorphic nature of these characters, common practice has been to term them adaptations. In the strict sense, however, the evidence of a true adaptive nature of these characters is still lacking. Adaptation is only one explanation for the correlated evolution of the morphological characters and epiphytism. In epiphytic habitats many complex structures such as a perfect hypnoid peristome, for example, may no longer be needed to ensure the effective dispersal, which might have led to their reduction (see also Vitt, 1981; Wilkens, 1988; Fong et al., 1995). It seems probable that, to some extent, the modified sporophytes could be the product of a relaxation of certain selective pressures in terricolous habitats. Another possible explanation for sporophytic specialization in epiphytes favors the role of natural selection. Sporophytic structures could be from a change in the direction of selection due to a shift from wind dispersal in terricolous habitats to water-assisted dispersal in epiphytic ones. In epiphytes the eco-

logical shift to new habitats has led to the considerable diversity in sporophyte structures (see Vitt, 1981). This diversity is also likely to be impacted by differences in edaphic habitats and the period of time species have occupied these epiphytic habitats and driven by even slight differences in ancestral morphology. Like the factors affecting the morphology of the epiphytic mosses, the role of random reductions versus adaptive evolution as driving forces may as well vary between species, thus, partly explaining the morphological variation.

Most studies dealing with the role of parallel or convergent characters in phylogenetic reconstruction have concentrated on showing their misleading effect within the study group where part of the terminals have, and part of them are lacking, the ecological conditions that are associated with the adaptive characters. This has often led to a neglect of these characters in phylogenetic analyses, hence ignoring part of the available information. As shown in this study, these characters do not necessarily lead to serious problems in phylogenetic reconstruction. It is important, however, to be aware of potentially correlated characters in data when performing analyses and to explore their effect.

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Appendix 1. Morphological characters and their coding.

Information for sporophytic characters of *Meteorium subpolytrichum* and *Floribundaria pseudofloribunda* was taken from the literature (Noguchi, 1976).

0. Growth from (0) stem prostrate, never ascending, sympodial branches arching; (1) stem ascending in apical parts; (2) stem pendent.
1. Branch foliage (0) terete; (1) complanate. Species with complanate foliage in our study never displayed the leaves arranged in two rows rather complanation was based on the orientation of base of leaves. Note that this character only refers to branch leaves, and especially in Meteoriaceae species with complanate branch leaf arrangement often have a primary shoot with julaceous foliage.
2. Branches (0) not curved; (1) curved. This refers to situations like *Brachythecium reflexum* and *Scorpiurium,* which have branches regularly aching when dry. In addition to Brachytheciaceae, similar curved branches can be observed in most *Meteorium* and *Papillaria* species, such as *M. papillarioides,* and *P. nigrescens.* Somewhat flexuose and only occasionally curved branches are coded as 0.
3. Whitish argent color in older parts of shoot (similar to that in *Rhynchostegium* species) (0) absent; (1) present.
4. Yellowish orange coloration of plants (0) absent; (1) present. In the specimens collected from shady habitats this coloration seems to be lacking. Hence a species was coded having this character if at least some of the specimens clearly had yellowish orange coloration. For example, *Chrysocladium retrorsum* specimens collected from tree trunks growing inside forest are usually dark green, while specimens from sunny roadside trunks and branches are perfectly carrot orange.
5. Black coloration in old parts of shoots (0) absent; (1) present. The black pigmentation at the basal portion of shoots seems to be common in the Meteoriaceae and Brachytheciaceae. It should not be confused with the brownish color resulting from the partial decomposition of older shoots.
6. Stem central strand (0) present; (1) absent. Among ingroup taxa only *Rhynchostegiella* and *Homalotheciella* completely lack the central strand. This character was coded as 1 in cases when in at least two transverse sections showed no traces of central strand, though in other sections a 1- to 3-celled strand could be discerned (*Zelometeorium, Rhynchostegiella teneriffae,* etc.). A clear, well-differentiated central strand is, however, present in some of the outgroups such as *Camptochaete arbuscula.* This character was always studied from older, well-developed portions of the primary shoot.
7. Stem cortex (0) 0 to 4 cell layers thick; (1) > 5 cell layers thick.
8. Stipe-like pattern (0) absent; (1) present. With stipe-like pattern we mean the proximal sympodial branches that have remote foliage with much smaller and strongly modified, usually deltoid, often reflexed leaves. This pattern is quite clear when the specimen is studied at a distance. It is not expressed in all specimens, but if it was observed in at least some, it was coded as 1. Stipe-like pattern is often present in the Meteoriaceae species in pendent shoots, which also seem to break easily and serve as asexual propagules.
9. Color of upper cells of axillary hair (0) hyaline; (1) brownish. If brownish, upper axillary hair cells were found even only occasionally, this character was coded as polymorphic (?).
10. Width of upper cells of axillary hair (0) essentially not wider than cells below; (1) wider and inflated compared with cells below. The species was coded as 1 even if only a few of axillary hairs corresponded.
11. Basal cells of axillary hair (0) brown and clearly darker compared to upper cells; (1) hyaline or clearly with lighter color than upper cells.
12. Axillary hairs (0) 2- to 4-celled; (1) with 5 or more cells, at least in some well-developed axillary hairs. Not only upper cells in axillary hairs were counted, but also the basal cells, which sometimes differ in shape and coloration from apical cells. The length of axillary hairs was counted from the best-developed hairs at the apex of the primary shoot. In older shoots axillary hairs are usually broken or even totally lost, and in secondary branches they often tend to be shorter.
13. Branching of axillary hairs (0) not branching; (1) often biseriate, but occasionally some hairs branched; (2) most of the axillary hairs frequently branched. Branching of axillary hairs seems to show some variation in most of species studied. According to our observations, it is present in all specimens only in some *Papillaria* species. Some Meteoriaceae, such as species of *Meteorium,* and *Chrysocladium,* however, often have partially biseriate axillary hairs with some found in most specimens. Species with biseriate axillary hairs also seem to have the occasional tendency to produce a few branched hairs.
14. Shape of apical cells in axillary hair (0) long and narrow; (1) whole axillary hair consisting of short, rounded-rectangular cells. Usually the most apical cell(s) in each axillary hair were considerably longer than cells below. In character state 1, cells along the entire axillary hair had similar shape, with only 1 to 3 of the basalmost cells (often also brown in color) slightly shorter than apical ones.
15. Axillary hairs frequently bent (0) absent; (1) present.
16. Pseudoparaphyllia of Brachytheciaceae/Meteoriaceae (B/M) type (0) acute to obtuse or truncate; (1) long-triangular or acuminate at least at some branch initials (excluding upper 2 mm, where branch initials were regarded as immature). If pseudoparaphyllia are not of B/M type, the character state was coded as inapplicable (?).
17. Pseudoparaphyllia type (0) B/M type absent; (1) B/M type present. In Brachytheciaceae and Meteoriaceae each branch primordia is covered by three leaf-like pseudoparaphyllia forming a unique regular pattern (see Ignatov, 1999).
18. Stem leaves (0) straight; (1) falcate (at least slightly).
19. Stem leaves (0) erecto-patent to patent; (1) erect to erect-appressed; (2) spread almost at 90° to shoot. This character refers to the position of the basal leaf relative to the stem, which is in our study groups mainly affected by the shape of the attachment line of leaf to stem. Usually species with more or less straight leaf attachment lines as well as species with long attachment line are more closely appressed to the stems. This character, like also the following one, was studied from dry specimens.

Appendix 1. Continued.

20. Branch leaves (0) erecto-patent to patent; (1) erect to erect-appressed; (2) spread almost at 90° to shoot. Especially in the pendent Meteoriaceae, the position of branch leaves differs clearly from that of primary shoot.
21. Reflexed leaves (0) absent; (1) present. Unlike the two previous characters, a reflexed leaf position is the result of the structure of the leaf itself rather than in leaf attachment.
22. Apex of stem leaves (0) acuminate with acumen no less than 1/5 of the leaf length; (1) acute, apiculate or very shortly acuminate; (2) piliferous.
23. Apex of branch leaves (0) acuminate; (1) acute, apiculate or very shortly acuminate; (2) piliferous.
24. Leaf acumen (0) not distinctly flexuose; (1) distinctly flexuose. In Meteoriaceae several species have usually a rather long and flexuous leaf acumen, e.g., *Aerobryidium filamentosum* and *Meteorium subpolytrichum.* Sometimes this state is present only in pendent, stipe-like branches with narrow specialized leaves, but this character was coded as present if a similar leaf acumen was found on other parts of the shoot.
25. Shape of stem leaves (0) plane; (1) with concave basal part; (2) entire leaf concave; (3) channeled from the base.
26. Stem leaves (0) not twisted in mid-leaf when dry; (1) twisted in mid-leaf. Leaves that are twisted in mid-leaf appear both in the Brachytheciaceae and Meteoriaceae. For example in Meteoriaceae, *Toloxis* have very regularly twisted leaves, which makes it easy to distinguish them from the Meteoriaceae.
27. Leaf plication (0) leaves without distinct longitudinal plicae; (1) leaves with distinct longitudinal plicae.
28. Leaf decurrences (0) shortly decurrent or decurrences almost lacking; (1) long decurrent. Even in cases where leaf decurrences are said to be lacking some faint trace of the structure can often be distinguished.
29. Shape of basal corners in leaves (0) not auriculate; (1) auriculate.
30. Serration in leaf margin (0) not to the base of leaf; (1) to the base.
31. Leaf margin of stem leaves (0) serrulate to serrate; (1) slightly serrulate to subentire, (2) serrate, at least some teeth backward reflexed.
32. Costa width in stem leaves (0) narrower than one third of leaf width; (1) wider than one third of leaf width. Species with the costa that is wider than one third of leaf width were rare in our study group. These species, including for example *Donrichardsia macroneuron,* are easy to separate from others.
33. Leaf costa (0) single; (1) double or none. In species with a very short double costa some leaves may lack a costa entirely. Due to this, these conditions are regarded here as one state.
34. Costa of stem leaf ending at the apex (0) without spine or uppermost cell of costa is slightly prorate with spine; (1) with spine.
35. Costa of branch leaf ending at the apex (0) without spine; (1) with spine.
36. Costa serrate at the apex (0) absent; (1) present. This character does not include the single spine at the branch or stem leaf apex.
37. Leaf margin at mid-leaf recurved as in *Chrysocladium* (0) absent; (1) present. In *Chrysocladium retrorsum* leaf margins at mid-leaf especially in dry specimen are distinctly recurved making this species easy to recognize. This character also appears in other Meteoriaceae, such as *Sinskea,* as well as in one outgroup species, *Rhytidiadelphus triquetrus.*
38. Leaf cell surface (0) smooth; (1) papillose with one papilla/cell; (2) pluripapillose.
39. Pattern of leaf cells papillosity (0) papillae arranged regularly in rows; (1) papillae scattered across leaf cell surface. Papillosity patterning has a diagnostic value among species previously included in *Papillaria.* In those cases where the leaf laminal cells have several papillae and these cells are very long and narrow, the possibility exists that the position of papillae in rows may be the artifact of very narrow cells.
40. Leaf laminal cells (0) not prorate; (1) cells abaxially prorate (sometimes only in branch leaves). Slightly protruding cell ends may lead to structures that look like papillae. Because the origin of these prorate "papillae" is clearly different from papillae discussed in the previous character, we considered it better to treat them as separate characters.
41. Alar cells in leaves (0) not different from juxtacostal cells; (1) as group of thin-walled cells, larger than adjacent juxtacostal cells; (2) as group of opaque, thick-walled cells, smaller than adjacent juxtacostal cells.
42. Alar group shape (0) triangular to subquadrate; (1) elongate along the leaf margin.
43. Basal laminal cells distributed in an opaque group across the base of leaves (0) present; (1) absent.
44. Sexual condition (0) autoicous; (1) dioicous including instances of the dioicous + rarely autoicous species; (2) phyllodioicous including instances of the dioicous + phyllodioicous species; (3) partly synoicous.
45. Striae present in lower part of exotome teeth (0) present; (1) absent.
46. Endostome segments (0) broadly and more or less regularly perforate; (1) at least some of the segments narrowly to not perforate; (2) segments absent or adherent to teeth.
47. Endostome basal membrane (0) greater than 1/4 of endostome length; (1) lesser than 1/4 of endostome length.
48. Endostome cilia length (0) more than 80% length of free portion of segment; (1) 30%–80% of free portion of segments; (2) less than 30% of free portion of segments, or cilia lacking.
49. Exostome enabling the spore dispersal (0) in the dry conditions; (1) in the humid conditions. *Aerolindigia* was coded as 0, as the exostome teeth in this species turn straight when wet, but form a high conus enclosing the capsule mouth. Spore dispersal is thus possible only in dry conditions, although hygroscopic movements resemble the species releasing their spores during rainy and foggy time periods.
50. Basal part of exostome (0) with red color; (1) without red color. According to observations by Hendenäs, this coloration seems to be typical for Brachytheciaceae species.

Appendix 1. Continued.

51. Exostome teeth transitional zone (0) short, 1 to 4 plates; (1) expanded, with more than 4 plates. The part of exostome where the ornamentation of outer exostomial plates is often changing from horizontally striate to cross-striolate or to a papillose surface, and exostome teeth sometimes has a "shoulder-like" due to the narrowing of teeth is important in regulating the hygroscopic movements of exostome teeth. Usually species with a short transition zone have more elaborate movements and hence the exostome is actively involved in the dispersal of spores.
52. Spore size (0) small (diameter never > 25 μm); (1) large (diameter at least sometimes > 25 μm).
53. Perichatial leaves (0) reflexed; (1) straight.
54. Operculum beak (0) twice or more the height of the rest of operculum; (1) present but shorter than twice the height of the rest of operculum; (2) conic. The shape of the operculum has been used as a delimiting character on the genus level in Brachytheciaceae.
55. Seta surface (0) smooth; (1) rough, at least a little in upper part.
56. Stomatal pore (0) round; (1) elongate.
57. Calyptra (0) naked; (1) hairy.
58. Seta length (0) longer than branches; (1) shorter than branches; (2) shorter than twice the urn length. Branch length is measured in place where branching is regularly pinnate and branches show rather stable lengths. We decided to compare the seta length in relation to branch length because of the extreme variation in plant size within our study group (for example, *Homalotheciella subcapillata* vs. *Pseudospiridentopsis horrida*).
59. Capsule orientation (0) inclined to horizontal; (1) erect. In the latter case the urn is sometimes clearly curved in the middle, as occasionally in *Leskea polycarpa*, but never curved below the mouth.

Appendix 2. Morphological character matrix.

Species	000000000011111111112222222222333333333344444444445555555555 01234567890123456789012345678901234567890123456789012345678901234567890123456789
Orthotrichum stramineum	10000010000?1000?000001100000011000000210O?10111?01??1101?21
Aerolindigia capillacea	20000001000100001100000003000000001100O?00?00001200111110011
Aerobryidium filamentosum	20000011000110010100000010000010000000 1?00?01011?0?111010?01
Aerobryum speciosum	20010101010010000102200000100010001100O?00?02000100001100010
Aerobryopsis wallichii	20000101000110010100000010000010000000 1?0200??????????????????
Amblytegium serpens	00000000001000O?000000001000001000000O?020000000100020100O
Ancistrodes genuiflexa	20000100001000O?000000011000001010000O?00?01011201011100011
Barbella flagellifera	20000010100110000100001100000000000000 1?020011121110100011
Barbella flagellifera	20000010100110000100001100000000000000 1?0200??????????????
Barbellopsis trichophora	21000101100111110100201010000001?1???00?020010112110111001 21
Bestia longipes	00100000000001000?00110110200000000011100?12101011111100000001
Brachytheciastrum collinum	00000000000?000001011011010000100000000?020000000000021000O
Brachythecistrum falcatulum	00000000000100000110000001000010000000?020000000000021000O
Brachytheciastrum velutinum	01000000000?000001100000010000100011100?020000000000021000O
Brachythecium acuminatum	10000000000000000101100001000000000000O?020010210000200001
Brachythecium albicans	10000000000000000111100001001001000000O?021010000000020000O
Brachythecium buchananii	10000000000000001100000001000001000000O?020010000000020000O
Brachythecium cirrosum	10000000000000000101102202000001000000O?020010000000011?000
Brachythecium complanatum	01000000000000000110000001000000000000O?010010000000021010O
Brachythecium erythrorrhizon	01000000000000001110000001011001000000O?0210??????????????
Brachythecium frigidum	10000000000?000001000000010110100001000?010010000000021000O
Brachythecium geheebii	10000000000000000100000001010000000100O?02001??????????????
Brachythecium glareosum	10000000000?000011011000010100010000000?020010000000020000O
Brachythecium laetum	10000000000000000101100001010001001100O?020010000000020000O
Brachythecium lamprocarpum	10000000000000001100000001000000000000O?011000000000020000O
Brachythecium mildeanum	10000000000000000100000001001001000000O?0?003000000000200000
Brachythecium rivulare	10000000000000000100001001001000000000O?010010000000021000O
Brachythecium ruderale	10000000000100001100000001000001000000O?020010000000020000O
Brachythecium rutabulum	10000000000000001100000001101000000000O?010000000010210000
Brachythecium salebrosum	10000000000000000110000001010000011000O?020000000000020000O
Brachythecium salebrosum	10000000000000000110000001010000011000O?020000000000020000O
Brachythecium subalbicans	10000000000000000100000001010000011000O?020000000000020000O
Brachythecium tommasinii	10000000000000000100000001000000000000O?011010000000011000O
Brachythecium turgidum	10000000000000000101100001010001000000O?020000000000020000O
Brachythecium udum	10000000000000000100000001000001000000O?010010000000020000O
Bryhnia scabrida	00000000000?000001010010020010100011000?110010000000010000O
Bryoandersonia illecebra	10000000110110000101101102000000000000O?020010000000000000O
Camptochaete arbuscula	10000001100000000?00?00110200000 1?1???00?020010000010002010O
Campylium stellatum	10000000O?000000?000010001000000?1???00?010010000010002010O0
Chrysocladium retrorsum	20001101000011100100000000010112000001 1?00?01111211010101 11
Cirriphyllum crassinervium	00000001000?100001011011010000000011100?021010000000000100O0
Cirriphyllum koponenii	10000000000000001100000001001000000000O?01001???????0??????
Cirriphyllum piliferum	00000000000100000101102001001000000000O?010010000000000100O0
Clasmatodon parvulus	00000000000000000101100002000001000000O?021001112111001000 01
Cryptopapillaria fuscescens	20100111100111000100000000110111000000 2000?01111210111 1?121
Cryptopapillaria penicillata	20001011000012100101101101000111000000 2100?0111?2?1??1?1??21
Ctenidium molluscum	01100010000100000?010000001000100?1???00?121010000000001000000
Diaphanodon blandus	20101110100100000100001100001001000000 1?00?01111?11011100021
Dolichomitriopsis diversiformis	10110000000000000?001101102000000000000O?021011112111010000 00
Donrichardsia macroneuron	00000001011000000110001101?000001000000?00?0??????????????
Donrichardsia patulifolia	00000000010000000110001102100000000000O?0100?0000000?0?10?00
Duthiella wallichii	10100001000100000100000000000010000000 1?020010001111000000 00
Eurhynchiadelphus eustegium	10100000000000000100000001000000011000?120020000000000000 00
Eurhynchiastrum pulchellum	00100000100100000100000101000010001110O?020020000000000000 00
Eurhynchium angustirete	00100000110000000100001102010010001110O?010020000000000000 00

Appendix 2. Continued.

Eurhynchium striatum	00100000110000000100000001010010001110 0?0100200000000000000
Floribundaria floribunda	21000010100100001102200000100000000000200100101 0?11100000121
Floribundaria pseudoflor-ibunda	21000000100100000102200000100000000000200100 1???????????0??21
Helicodontium capillare	00000000000000000100001101000000011100 ?0210001121100011001 1
Homalothecium aureum	10100001000110001101100001010000001110 0?0201100010110121000 0
Homalothecium fulgescens	10100000000010001101100001010010001100 0?0201200000100111000 0
Homalothecium laevisetum	10000000010110000101100001010010001110 0?0201202121110100010 1
Homalothecium lutescens	10000000000110001101100001010010001100 0?0201200010100111000 0
Homalothecium megaptilium	10000001000110001101100001010000001110 0?0201200000000121000 0
Homalothecium nuttalii	10100000000110001101100001010012001110 0?0201200021010121000 0
Homalothecium philippeanum	1000000101 0?1000010110000101000000000 0?0201211211101100001
Homalothecium sericeum	10100000000110000101100001010012001100 0?0201101101011100001
Homalotheciella subcapillata	10010010000100000100000001000000000000 0?0210002121110101000 1
Hylocomium splendens	00000011100010000?0000001010000000?1???00?11101000000000100000
Hypnum cupressiforme	01000000000000000?0100000100000 1?1???00?020010000010010000000
Isothecium alopecuroides	00100011100010000?0011011020000010000000?0200111111101100001
Kindbergia praelonga	10000000100010001100010000001000000011 00?11001???????0?????0
Lembophyllum cladestinum	10000000000000000?0011011020000010000000?0200101020101010000 1
Lepyrodontopsis trichophylla	20000010100000000?0000011000000 10?1???00?00?01111211101?0?001
Leskea polycarpa	00100010000000000?0111011010000010000001?0210001121110120100 1
Leucodon sciuroides	10100011000000000?0011000010100 00?1???00?021011212111110?001
Lindigia debilis	20000000000011000?0000000001000100000000?00?01011211101100011
Meteorium crispifolium	10000101000110100100000010000110000000 2000?0??????????????
Meteorium papillarioides	20100101101010100101100010000110000000 1?00?01??????????????
Meteorium polytrichum	20100111000001?10010110001101011100000 01?00?0111121?101100111
Meteorium subpolytrichum	20100101100011100101102210000110000000 1?00?01?????1?0????11
Meteoridium remotifolium	20000010000010000102202200100000000000 0?00?0100120010100002 1
Meteoriella soluta	20000111000000000?0000100010001 02?1???00?00?011???????0?0??11
Meteriopsis squarrosa	20100101000110000100010000000010000000 1?00?0101111?001000111
Myuroclada maximowiczii	10000000000000000101101102000000000000 0?0210100000000100000
Neckera complanata	01000010100000000?0000011000000 01?1???00?020010112110?1000?01
Neobarbella comes	11000010000001000?0000000010000 01?1???00?00?01111211101000011
Neodicladiella pendula	20000010100110000100001100000000000000 2002001111211101100021
Neodicladiella pendula	20000000100110000100001100000000000000 200200??????????????
Okamuraea brachydictyon	00000000010100000101100001000001000000 0?0210102021011110010 1
Oxyrrhynchium hians	01000000101000000100000100100000011000 ?0100100000000010000
Oxyrrhynchium pumilum	01000000101000000100001100100000011000 ?00?0100000000010000
Oxyrrhyncium savatieri	01000000101000000100000100100000011100 ?0100100000000010000
Oxyrrhyncium vagans	01100000101000000100001100100000011000 ?0100100000000000000
Palamocladium euchloron	10100001010010000111100001010112000000 0?0200200021011000000 0
Palamocladium leskeoides	10?00001010010000101100001010110000000 0?0200200211000000001
Papillaria crocea	20101010100012100101101100000111000000 2100?01111210111001?111
Papillaria deppei	20100110000011100100000010 0?0110000000?000?01111211111000111
Papillaria flavo-limbata	20101011100012100101101100000111000001 2100?01111210111100111
Papillaria intricata	20000110100112000100001100100110000000 2000?011?1?1?1?1?00111
Papillaria leuconeura	2010101?10001210010110110100011100000021 00?01111211101100121
Papillaria nigrescens	20101111100011100101101000000111000000 2000?01111211 0?100?101
Papillaria nitens	20100010100012100100001110000010000001 2000?01111210101 01?101
Platyhypnidium austrinum	00000000010010000110001100100000000000 ?0100000000000010000
Plagiothecium denticulatum	01000000000000000??0000110010100 1?1???00?00?0000000100020 1000
Platyhypnidium riparioides	00000000010?10000110001100100010000000 ?0100000000000000000
Plasteurhynchium striatulum	00100000110010000100000000000010001100 0?00?1100000000000000
Pseudospiridentopsis horrida	10000111000110010100010010000010000000 1?00?01?????????????11
Pseudotrachypus attenuata	21000111000110011102200010000010000000 1?00?0101010011111100 1
Pseudotrachypus martinicensis	21000110000110011102200010000011000000 1?00?0??????????????
Pseudoscleropodium purum	10000000010010000101101102000000000000 0?0100100000001200000
Pyrrhobryum latifolium	10000000000001000?0100011000000000011100 ?00?010?000000120?000
Remyella brachypodia	20000000000100001102200001000010000000 0?0110001010011001000 1
Rhynchostegiella macilenta	00000010100000000100000000000000 1000000?00?0000000000101000 0
Rhynchostegiella teneriffae	00000010100000000100000000000000 1000000?00?0000000000101000 0
Rhynchostegiella tenella	00000010000000000110000000300001100000 00?0100000010000000000
Rhynchostegium arcticum	01000000010000000101101102000000000000 0?0100?????????????00
Rhynchostegium confertum	01010000010010000100000000000010001000 0?0100000100000000000
Rhynchostegium murale	01000000010000000101101102000000000000 0?0100000000000000000

Appendix 2. Continued.

Rhynchostegium pallidifolium	0101000001001000010000000010001000000?0100000000000000000
Rhynchostegium psilopodium	0000000001000000010000000100001000011000?0100000000000000000
Rhynchostegium rotundifolium	0000000000000000010000110010000000000000?0100000000001000000
Rhynchostegium serrulatum	0101000001001000010000000010001000000000?0100000000000000000
Rhytidiadelphus triquetrus	1000000110011000?0000100020001001?1???10?1200100000100201000
Rozea subjulacea	1010001000010000?0011011020000000000000?0200011211111100001
Rozea andrieuxii	1010001000010000?0011011020000010000000?0200011211101100001
Sciurohypnum flotowianum	0000000100010000010110000100000100000000?0201101000000110000
Sciurohypnum glaciale	0010000000010000010110110100100100000000?0200000000000210000
Sciurohypnum hylotapetum	0101000000011000010000000000100000000000?0100100000000210000
Sciurohypnum latifolium	0000000000010000010110110100100100000000?0100000000000210000
Sciurohypnum oedipodium	0100000000010000010000000010100000001000?0100000000000210000
Sciurohypnum ornellanum	0010000000010000010110110200100000000000?0100000000000210000
Sciurohypnum plumosum	0000000000010000010110000100001000001000?0201000000000210000
Sciurohypnum populeum	0000000000010000010110000100000100000000?0201000000000210000
Sciurohypnum reflexum	0010000000010000010111000110100000000000?0100000000000210000
Sciurohypnum obtusifolium	1000000000011000010110110200000100000000?0200100000000210000
Scorpiurium circinatum	1010000010001000010110110100000000011100?0211100000000100000
Scorpiurium deflexifolium	1010000010001000010110110100000000011100?0211??????????????
Sinskea flammea	2100110010010000010020000000001200000020000?0111111010110021
Sinskea phaea	2100110010010000010020001000001200000020000?0111121111110?121
Squamidium brasiliense	2000011000001000010110001100100100000000?0200111121111110012 1
Toloxis imponderosa	2000011000011100010000110011011110000120000?01??????????????
Toloxis semitorta	2000010010011000010000110011011100000120000?0111121111100001 1
Trachycladiella aurea	2100001000011000110?2000001000100000012000?0101020010011002 1
Trachypus bicolor	1000010000010001?0000000000000110000002000?0111021111001010 1
Trachypodopsis serrulata	0000000000010001010000111001011000000001?0200101121111111010
Unclejackia longisetula	0000000000000000110000221100000100000000?0110001120011021000 1
Weymouthia mollis	2000001010000000?0000011020000001?1???00?0200100010101000001 1
Zelometeorium patulum	2000011000000000010001001010001000000000?0200111121011001012 1

Appendix 3. Commands used in all POY analyses.

poypc -maxtrees 5 -holdmaxtrees 100 -random 20 -multibuild 14 -gap 1 -treefuse -fuselimit 25 -fusingrounds 3 -gap 1 -driftspr -time -numdriftspr 5 -drifttbr -numdrifttbr 5 -slop 0 -checkslop 10 -seed -1 -noleading -fitchtrees -indices -norandomizeoutgroup *datafiles*.txt > *results*.out 2 > *errofile*.runlog

PHYLOGENY AND BIOGEOGRAPHY OF *PLAGIOCHILA* (PLAGIOCHILACEAE) BASED ON NUCLEAR AND CHLOROPLAST DNA SEQUENCES[1]

Henk Groth[2], *Melanie Lindner*[2], *and Jochen Heinrichs*[2]

Abstract

Cladistic analyses based on the chloroplast protein coding gene *rps*4 and the internal transcribed spacer region of nuclear ribosomal DNA (ITS1-5.8S-ITS2) were conducted to investigate phylogenetic relationships among species of *Plagiochila* and to reconstruct the ranges of natural species groups within this genus. Data from each DNA region were considered separately and in combined analyses. Maximum likelihood and parsimony analyses led to largely similar topologies, but the analyses of the combined data set contained more informative characters and more robust bootstrap or Bayesian support. Natural species groups of *Plagiochila,* here assigned to sections, usually cover several floristic kingdoms. The analyses reveal close relationships between the *Plagiochila* species from the Neotropics and Atlantic Europe. Some groups are pantropical in distribution. Several species from South America are closely related to species from New Zealand. The molecular topologies lead to the following new synonyms: *P.* sect. *Banksianae* [= *Plagiochila* sect. *Alternantes*]; *P.* sect. *Deltoideae* [= *P.* sect. *Durae*]; *P.* sect. *Zonatae* [= *P.* sect. *Peculiares*], *P.* sect. *Carringtoniae* [= *P.* sect. *Poeltiae*], *P.* sects. *Parallelae* and *Subtropicae* [= *P.* sect. *Vagae*]. The pantropical *Plagiochila* sects. *Vagae* and *Cucullatae* are nested in a large tropical Asian-Holarctic clade, implying that this group descended from Asian ancestors. Due to the frequent occurrence of homoplasy, it is not possible to identify natural species groups by morphology alone. The molecular data support a hierarchical subdivision of *Plagiochila,* however, deeper nodes are not supported by morphology.

Key words: biogeography, ITS, molecular phylogeny, *Plagiochila,* Plagiochilaceae, *rps*4.

Plagiochila (Dumort.) Dumort., with over 1600 published binomials worldwide (Inoue, 1984), is the largest genus of hepatics. The genus is defined by its laterally compressed perianth with a dorsal keel usually longer than the ventral one, dioicous condition, succubous leaves, and alternating foliation. Species of *Plagiochila* occur worldwide with the exception of the Antarctic mainland. Centers of diversity, however, are the humid tropics where *Plagiochila* species are found from the lowlands to above 4000 m.

Many species reach lengths of more than 10 cm and can build large mats. Especially in mountainous rainforests, *Plagiochila* belongs to the most common bryophytes, growing on soil, rock, as well as epiphytically from the trunk bases up into the canopy. Accordingly, *Plagiochila* is collected by many botanists and thousands of specimens are stored in the herbaria. However, *Plagiochila* is notorious for variation in gametophytic characters and is considered to be "perhaps the most difficult" genus of the Hepaticae (Schuster, 1980: 341). Despite the frequency of occurrence, knowledge about species delimitation, as well as species relationships, is still limited.

Taxonomy and Distribution of *Plagiochila* Species

Early authors (e.g., Gottsche, 1863–1867; Stephani, 1901–1906, 1917–1925) tried to cope with

[1] Many botanists sent material for molecular investigation or allowed us to extract DNA from specimens housed in their herbaria: We thank Hironori Deguchi (Hiroshima), Wolfgang Frey (Berlin), Ingo Holz (Göttingen/Greifswald), David Long (Edinburgh), Jochen Müller (Jena), Tanja Pfeiffer (Berlin), Tamás Pócs (Eger), Michelle Price (Geneva), Elena and Uwe Reiner-Drehwald (Göttingen), Carsten Renker (Leipzig), David Rycroft (Glasgow), Alfons Schäfer-Verwimp (Herdwangen-Schönach), and Tomio Yamaguchi (Hiroshima) for their generous help. Thanks to S. Rob Gradstein (Göttingen) and David Rycroft for fruitful discussions and to Rosemary Wilson (Göttingen) for improving the English. Christine Davis (Durham) and John Wheeler (Berkeley) kindly provided their *rps*4 sequencing protocols. Financial support by the German Science Foundation (grant DFG HE 3584/1) is gratefully acknowledged.

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the bewildering diversity in *Plagiochila* by creating numerous binomials, often known only from the type and with unclear morphological delimitation. Study of larger numbers of specimens usually makes the case for wider species concepts and extension of ranges. Jones (1962), Grolle (1989), and Vanden Berghen (1981) each synonymized 15 binomials under the African *Plagiochila integerrima* Steph. and *P. terebrans* Lindenb. So (2001a) placed 19 binomials in the synonymy of the Asian *P. sciophila* Lindenb. Heinrichs and Gradstein (2000) treated 20 binomina as synonyms of the American *P. raddiana* Lindenb. The overall impression is that the number of described binomina is at least three to four times higher than the number of species themselves. Accordingly, So and Grolle (2000) estimated the number of *Plagiochila* species to be 400–450 worldwide.

Until very recently, the so-called geographical species concept dominated *Plagiochila* taxonomy. However, several studies combining molecular, phytochemical, and morphological data (e.g., Heinrichs, 2002; Rycroft et al., 2002; Groth et al., 2003, 2004; Heinrichs et al., 2004a), revealed the occurrence of intercontinental ranges or other large disjunctions of many *Plagiochila* species.

Subdivision of *Plagiochila* Above the Species Level

Besides the taxonomy and distribution of the species, the subdivision of the genus *Plagiochila* into natural groups is subject to controversy. The first attempt to classify *Plagiochila* was published by Lindenberg (1839–1844). He recognized six sections worldwide, based on characters of the dried gametophytes including perianth and leaf shape as well as branching pattern. This subdivision was accepted by Gottsche et al. (1844–1847). Spruce (1884–1885) proposed a new concept for the Neotropical Plagiochilae. Based upon branching pattern and perianth position, he split the genus into Ramiflorae and Cauliflorae, with two and three sections, respectively. In his treatment of *Plagiochila* in tropical Africa, Jones (1962) principally followed Spruce; however, he separated the Ramiflorae from the Cauliflorae by the frequent occurrence of lateral-terminal branches. Inoue and Schuster (1971) also subdivided *Plagiochila* into Cauliflorae (with 9 sections in 2 groups) and Ramiflorae (with 3 sections in 2 groups). However, their Cauliflorae have perianths at the main stems, whereas their Ramiflorae are characterized by plants with perianths on short lateral branches. Schiffner (1900a, b) improved the classification of the tropical Asian representatives of *Plagiochila* by spreading the species over seven sections defined by gametophytical characters such as leaf position, cell pattern and dentation, underleaf structure, branching, and perianth position.

Stephani (1901–1906) divided *Plagiochila* into the rank free Ampliatae and Patulae, based on the shape of the ventral leaf base. Within these units, he arranged the species according to their origin. Dugas (1929) chose a similar approach. Independent from their provenance, she distributed the species of *Plagiochila* over the rank free Ligulatae, Rotundifoliae, and Trigonifoliae, which were solely based on leaf shape.

Only two years after Dugas's treatment, Carl (1931a) published his important subdivision of *Plagiochila*. He excluded *Plagiochila dendroides* (Nees) Lindenb. (as *Chiastocaulon dendroides* (Nees) Carl) from the genus because of differences in branching (Carl, 1931b). The remaining taxa were spread over the three *Plagiochila* subgenera *Cucullifoliae* Carl, *Oppositae* Carl, and *Eu-Plagiochilae* (Carl, 1931a). The latter group more or less agrees with the current concept of *Plagiochila*. *Plagiochila* subgen. *Oppositae* is now treated as *Plagiochilion* S. Hatt. (Hattori, 1947). *Plagiochila* subgen. *Cucullifoliae*, erected to genus level (i.e., *Szweykoswkia* Gradst. & M. Reiner) by Gradstein and Reiner-Drehwald (1995) has recently been placed in the synonymy of *Plagiochila* sect. *Hylacoetes* Carl (Heinrichs, 2002).

In a first step, Carl (1931a) divided the species of subgenus *Eu-Plagiochila* based on their distribution in three floristic kingdoms (Neotropics, Paleotropics, Austral-Antarctic; Africa not treated). In a second step, he defined 50 sections that were based on several characters of the dried gametophytes, including leaf cell pattern, density of foliation, branching, vegetative reproduction, position of the androecia and gametangia, as well as dentation of the male bracts. These sections were usually restricted to a single floristic kingdom.

Schuster (1959) criticized Carl's division based on geographical criteria and the often very small sections, which can be monospecific after elimination of the synonyms (e.g., Müller et al., 1999). Other authors improved Carl's concepts by the introduction of new morphological characters. Inoue and Schuster (1971) and Heinrichs et al. (2000) demonstrated the value of oil body characters for the taxonomy of *Plagiochila*. Unlike Carl (1931a), Inoue and Schuster (1971), Inoue (1984), and Heinrichs (2002) used not only gametophyte but also sporophyte characters.

However, despite its deficiencies, Carl's subdi-

vision—based on the combination of many putatively monophyletic binomina—was a milestone toward a natural system of *Plagiochila.* Later works (e.g., Schuster, 1959; Inoue, 1984) usually affected the separation or summarization of supraspecific taxa recognized by Carl (1931a) or the exclusion of particular groups from *Plagiochila,* e.g., *Acrochila* R. M. Schust. or *Xenochila* R. M. Schust.

Substantial changes of Carl's concepts primarily arose from the work of Inoue (1984), who raised two sections to subgenera (*Plagiochila* sect. *Peculiares* Schiffn. → *P.* subgen. *Paraplagiochila* Inoue, *P.* sect. *Cucullatae* Schiffn. → *P.* subgen. *Metaplagiochila* Inoue), lowered *Chiastocaulon* to a subgenus of *Plagiochila,* and established the subgenus *Pleurochila* for a single New Zealand species, *P. pleurata* (Hook. f. & Taylor) Gottsche, Lindenb. & Nees. Five subgenera including the type subgenus arose from this study of which one (the subgenus *Plagiochila*) includes the vast majority of species. Inoue's hierarchical subdivision of *Plagiochila* into subgenera and sections was accepted by some recent authors (e.g., Grolle & So, 1999; So, 2001a; So & Grolle, 2001).

Several molecular studies of predominantly Neotropical and Holarctic Plagiochilaceae led to extensive rearrangements of the previous subdivisions. Heinrichs (2002) lowered the two Neotropical genera *Steereochila* Inoue and *Szweykowskia* Gradst. & M. Reiner to synonyms of *Plagiochila* sect. *Hylacoetes,* based on analyses of nr ITS sequence variation. Heinrichs et al. (2004b) placed *Rhodoplagiochila* R. M. Schust. in the synonymy of *Plagiochila bifaria* (Sw.) Lindenb. Groth and Heinrichs (2003) used cp *rps*4 and nr ITS sequences for a reinstatement of the genus *Chiastocaulon,* which is placed in a clade with *Pedinophyllum* and *Plagiochilion,* sister to *Plagiochila.* Groth et al. (2002, 2003), Heinrichs (2002), and Heinrichs et al. (2002a) merged several of the morphologically defined sections as an outcome of maximum likelihood analyses of ITS sequence alignments. They demonstrated that many morphological characters are homoplastic with respect to *Plagiochila* and that careful integration of molecular data is necessary for a natural subdivision of the genus. Furthermore, they provided evidence that Carl's (1931a) geographical subdivision of *Plagiochila* is outdated.

The aim of the present paper is to evaluate the subgeneric and sectional concepts of the earlier authors by means of molecular markers and to reconstruct the ranges of several monophyla within *Plagiochila.*

Materials and Methods

Taxon sampling

Sixty-four species of *Plagiochila* and one variety from major parts of the generic range were sampled based on available material and to represent the morphological diversity of the group. According to the results of Groth and Heinrichs (2003), *Chiastocaulon dendroides* (Nees) Carl, *Pedinophyllum interruptum* (Nees) Kaal., and *Plagiochilion mayebarae* S. Hatt. (Plagiochilaceae) were chosen as the outgroup.

Eighteen sequences of the nr ITS region (ITS1-5.8S-ITS2) and 41 cp *rps*4 sequences were newly generated for this study. Fifty sequences of the ITS region and 27 *rps*4 sequences were taken from Groth & Heinrichs (2003), Groth et al. (2002, 2003, 2004), Heinrichs (2002), Heinrichs et al. (2002a, b), Renker et al. (2002), or Rycroft et al. (2003). Vouchers are deposited at GOET or HIRO (Table 1).

DNA extraction, PCR amplification, and sequencing followed the protocols described in Groth and Heinrichs (2003).

Phylogenetic analyses

The 68 sequences of the ITS region as well as the *rps*4 sequences were aligned manually in BioEdit version 5.0.9 (Hall, 1999). Within the ITS alignment, ambiguously aligned sites and the often incomplete end of the ITS2 spacer were excluded. Primer regions of the *rps*4 sequences were also excluded. The lacking ITS2 sequence of *Plagiochila bicuspidata* Gottsche was scored as missing. Both alignments were combined.

Phylogenetic trees of the separate and the combined data sets were inferred using maximum likelihood (ML) and maximum parsimony (MP) criteria as implemented in PAUP* version 4.0b10 (Swofford, 2000).

ML analyses

To decide on the nucleotide substitution model with the smallest number of parameters that best fits the data, the program Modeltest 3.06 (Posada & Crandall, 1998) was used. Based on the results of the tests, the models selected by the hierarchical likelihood ratio tests were TrN + I + G for the ITS data set, TVM + G for the *rps*4 data set, and GTR + I + G for the combined data set. Maximum likelihood analyses (in each case with the appropriate model) were implemented as heuristic searches with 10 random-addition sequence replicates. The confidence of branching was assessed using Mr-

Table 1. Geographic origins, voucher numbers, and GenBank/EMBL accession numbers of the investigated taxa. Accession numbers of new sequences are in bold.

Taxon	Origin	Voucher	ITS	*rps*4
Chiastocaulon dendroides (Nees) Carl	Japan	*Ohnishi 5770* (HIRO)	AY438233	AY438213
Pedinophyllum interruptum (Nees) Kaal.	United Kingdom	*Rycroft 020907* (GOET)	AY438234	AY438216
Plagiochila adiantoides (Sw.) Lindenb.	Costa Rica	*Heinrichs et al. 4314* (GOET)	AJ422027	AY438204
P. aerea Taylor	Costa Rica	*Heinrichs et al. 4321* (GOET)	AJ422028	AY438205
P. alternans Lindenb. & Gottsche	Bolivia	*Heinrichs et al. GP16* (GOET)	**AY550130**	**AY547691**
P. arbuscula (Lehm. & Lindenb.) Lindenb.	Japan	*Yamaguchi 18280* (HIRO)	**AY550131**	**AY547692**
P. asplenioides (L.) Dumort.	Germany	*Heinrichs & Groth 4339* (GOET)	AJ414629	**AY547693**
P. banksiana Gottsche	New Zealand	*Frey & Pfeiffer 98-T121* (GOET)	**AY550132**	**AY547694**
P. bantamensis (Reinw., Blume & Nees) Mont.	Japan	*Yamaguchi 16890* (HIRo)	AY27560	**AY547695**
B. biscuspidata Gottsche	Costa Rica	*Holz CR 99-787* (GOET)	**AY550133**	**AY547696**
P. bifaria (Sw.) Lindenb.	Ecuador	*Holz EC-01-416* (GOET)	AJ422010	AY438206
P. britannica Paton	United Kingdom	*Rycroft 00015* (GOET)	AY275162	AY438207
P. buchtiniana Steph.	Bolivia	*Groth s.n.* (GOET)	AJ413306	AY438208
P. carringtonii (Balf.) Grolle ssp. *lobuchensis* Grolle	Bhutan	*Long 28857* (GOET)	AJ414631	AY438209
P. chonotica Taylor	Chile	*Holz & Franzaring CH-00-108* (GOET)	**AY550134**	**AY547697**
P. cristata (Sw.) Lindenb.	Costa Rica	*Heinrichs et al. 4192* (GOET)	AJ422015	AY438210
P. cucullifolia Jack & Steph. var. *anomala* J. Heinrichs & Gradst.	Ecuador	*Holz EC-01-558* (GOET)	AY330711	AY438211
P. deflexa Mont. & Gottsche	Costa Rica	*Heinrichs et al. 4160*	AJ416083	**AY569439**
P. delfexirama Taylor	Costa Rica	*Heinrichs et al. 11* (GOET)	**AY550135**	**AY547698**
P. deltoidea Lindenb.	New Zealand	*Frey 98-T116* (GOET)	**AY550136**	**AY547699**
P. disticha (Lehm. & Lindenb.) Lindenb.	Ecuador	*Holz EC-01-436* (GOET)	AJ422014	AY438214
P. diversifolia Lindenb. & Gottsch	Ecuador	*Holz EC-01-17* (GOET)	AJ413308	AY438215
P. dura De Not.	Chile	*Drehwald 3190* (GOET)	**AY550137**	**AY547700**
P. ensiformis Taylor	Ecuador	*Sauer & Gradstein MS-E 115, BEGP 149* (GOET)	AY275163	**AY547701**
P. exigua (Taylor) Taylor	Ireland	*Hakelier s.n.* (GOET)	AF539461	**AY547702**
P. flexuosa Mitt.	Japan	*Kurita 147* (HIRO)	**AY550138**	**AY547703**
P. frondescens (Nees) Lindenb.	Indonesia	*Schäfer-Verwimp 20704* (GOET)	AY438237	AY438219
P. fruticosa Mitt.	India	*Long 23002* (GOET)	AY438235	AY438217
P. fuscolutea Taylor	Costa Rica	*Heinrichs et al. 4400* (GOET)	AJ416086	**AY547704**
P. gymnocalycina (Lehm. & Lindenb.) Mont.	Bolivia	*Drehwald & Reiner-Drehwald 4729* (GOET)	AY390531	AY438218
P. hakkodensis Steph.	Japan	*Yamaguchi 12271* (HIRO)	AY275164	**AY547705**
P. heterophylla Lehm.	United Kingdom	*Rycroft et al. 97006* (GOET)	AY275165	**AY547706**
P. integerrima Steph.	Malawi	*O'Shea M7552a* (GOET)	AY275166	**AY547707**
P. lastii Mitt.	Comores	*Pócs et al. 9273/U* (GOET)	**AY550139**	**AY547708**
P. longiramea Steph.	Bolivia	*Heinrichs et al. 4071* (GOET)	AF539463	**AY547709**
P. macrostachya Lindenb.	Ecuador	*Holz EC-01-80* (GOET)	AJ422016	**AY547710**
P. magna Inoue	Japan	*Kurita 258* (HIRO)	AY275167	AY438221
P. orbicularis (S. Hatt.) S. Hatt.	Japan	*Kurita 32* (HIRO)	AY275168	AY438222
P. ovalifolia Mitt.	Japan	*Ohnishi 5723* (HIRO)	AY275169	**AY547711**
P. ovata Lindenb. & Gottsche	Costa Rica	*Heinrichs et al. 4158* (GOET)	AJ422017	**AY547712**
P. patentissima Lindenb.	Brazil	*Gradstein 3703* (GOET)	**AY550140**	**AY547713**
P. patriciae J. Heinrichs & H. Anton	Costa Rica	*Holz CR-00-176* (GOET)	AY275170	**AY547714**

Table 1. Continued.

Taxon	Origin	Voucher	ITS	*rps*4
P. patzschkei Steph.	Ecuador	*Holz EC-01-389* (GOET)	AJ422018	**AY547715**
P. peculiaris Schiffn.	Bhutan	*Long 28832* (GOET)	**AY550141**	**AY547716**
P. poeltii Inoue & Grolle	India	*Long 22802* (GOET)	**AY550142**	**AY547717**
P. porelloides (Nees) Lindenb.	Germany	*Heinrichs & Groth 4340* (GOET)	AJ414633	**AY547718**
P. pulcherrima Horik.	Japan	*Ohnishi 5771* (HIRO)	AY438239	AY438223
P. punctata (Taylor) Taylor	United Kingdom	*Rycroft 01013* (GOET)	AJ413174	**AY547719**
P. raddiana Lindenb.	Ecuador	*Holz EC-01-45* (GOET)	AJ422020	AY438225
P. renitens (Nees) Lindenb.	Malaysia	*Schäfer-Verwimp 18736/A*	**AY569440**	**AY569441**
P. retrorsa Gottsche	Costa Rica	*Heinrichs et al. 4154* (GOET)	AJ422021	**AY547720**
P. retrospectans (Spreng.) Lindenb.	New Zealand	*Frey & Pfeiffer 98-T88* (GOET)	**AY550143**	**AY547721**
P. rutilans Lindenb.	Bolivia	*Groth 101* (GOET)	AJ416081	AY438226
P. rutilans var. *moritziana* (Hampe) J. Heinrichs	Ecuador	*Holz EC-01-404* (GOET)	AJ416080	**AY547722**
P. sandei Sande Lac.	Indonesia	*Gradstein 990* (GOET)	AJ414634	AY438227
P. satoi S. Hatt.	Japan	*Ohnishi 5720* (HIRO)	**AY550144**	**AY547723**
P. sciophila Lindenb.	Japan	*Ohnishi 5400* (HIRO)	AY275171	**AY547724**
P. semidecurrens (Lehm. & Lindenb.) Lindenb.	Nepal	*Long 21348* (HIRO)	AY275173	AY438228
P. spinulosa (Dicks.) Dumort.	Belgium	*Dauphin et al. 3811* (GOET)	AY275173	**AY547725**
P. stricta Lindenb.	Costa Rica	*Heinrichs et al. 4401* (GOET)	AJ416646	AY438229
P. subplana Lindenb.	French Guiana	*Holz FG-00-32* (GOET)	AY275174	AY438224
P. subtropica Steph.	Nepal	*Long 17359* (GOET)	**AY550145**	**AY547726**
P. superba (Spreng.) Mont. & Nees	Costa Rica	*Heinrichs et al. 4175* (GOET)	AJ416084	AY438230
P. tabinensis Steph.	Costa Rica	*Heinrichs et al. 4156* (GOET)	AJ422026	AY438231
P. trabeculata Steph.	Japan	*Kurita 257* (HIRO)	**AY550146**	**AY547727**
P. trichostoma Gottsche	Costa Rica	*Heinrichs et al. 4324* (GOET)	AJ416082	**AY547728**
P. turgida Herzog	Ecuador	*Holz EC-70* (GOET)	AJ422024	**AY547729**
Plagiochilion mayebarae S. Hatt.	Japan	*Ohnishi 55883* (HIRO)	AY438238	AY438220

Bayes (version 3.0b4; Huelsenbeck & Ronquist, 2001), a program for Bayesian inference of phylogeny. The Modeltest 3.06 output values were applied to the command block of MrBayes. Five hundred thousand generations were calculated of which every tenth was saved, resulting in 50,000 saved trees. Trees collected before the likelihood score had stabilized were deleted. The remaining 40,000 trees with a stable likelihood score were condensed to a majority rule consensus tree. Branch support of the consensus tree, referred to as posterior probabilities, was regarded as significant when exceeding 0.95 (Larget & Simon, 1999).

MP analyses

Maximum parsimony analyses were performed with the following options implemented: heuristic search mode with 1000 random-addition-sequence replicates, tree bisection-reconnection branch swapping (TBR), MULTrees option on, and collapse zero-length branches off. All characters were treated as equally weighted and unordered. Non-parametric bootstrap support (Felsenstein, 1985) was estimated with full heuristic searcher, 1000 bootstrap replicates, and 10 random addition sequence replicates per bootstrap replicate, TBR swapping, MULtrees option on, collapse zero-branches off, and by saving all trees.

Congruence between the trees generated from the ITS and the *rps*4 data set respectively was inferred by inspecting Bayesian support values above 0.94 (Larget & Simon, 1999) in ML analyses and bootstrap scores above 70% (Hillis & Bull, 1993; Mason-Gamer & Kellogg, 1996) in MP analyses.

NAMING OF CLADES

Current systems of formal ranking allow the hierarchical classification of plant genera into subgenera, sections, subsections, series, subseries, and species (Art. 4.2 ICBN, Greuter et al., 2000). Well-resolved molecular trees may include more nodes than covered by the above grades. Although Art. 4.3 of the ICBN allows the establishment of further ranks, we hesitate to propose a new deeply hierarchical subdivision of *Plagiochila* based on our results because (1) the number of (well-supported)

nodes may differ depending on the analytical method of a given data set; (2) data set partitions may lead to somewhat different topologies; and (3) inclusion of further taxa as well as expansion of the marker set may lead to changes in the topologies. Therefore, we applied only the most widely used rank for species groups below *Plagiochila*—the section—and summarize monophyla of morphologically similar binomina under a sectional name irrespective of their sister relationships.

Results and Discussion

The alignment of the ITS1-5.8S-ITS2 sequences and the *rps*4 sequences of 68 taxa (including 3 outgroup taxa) resulted in 1230 sites (ITS: 666; *rps*4: 564), which were used for the phylogenetic analyses. Of the 1230 characters, 643 (ITS: 319; *rps*4: 324) were constant, 197 (ITS: 106; *rps*4: 91) variable but parsimony uninformative, and 390 characters (ITS: 241; *rps*4: 149) were parsimony informative.

MP and ML analyses of the separate and combined data sets

Parsimony and maximum likelihood analyses of the separate ITS and *rps*4 data sets led to similar topologies (Figs. 1, 2; MP trees of the separate data sets not depicted). The ML trees derived from the nuclear and the chloroplast markers differ in the position of the *Alternantes* clade, which represents the basalmost *Plagiochila* lineage in the *rps*4 tree (Fig. 1) but is placed sister to the *Glaucescentes* in the ITS tree (Fig. 2). However, the latter topology is not well supported. Another difference is found in the position of *P. peculiaris* Schiffn. In the ITS tree, the species is placed at the base of a well-supported clade with representatives of *P.* sect. *Zonatae* Carl (synonymized to *P.* sect. *Peculiares* Schiffn., see below). In contrast, the *rps*4 data set does not resolve a clear sister relationship of this species within a larger clade including the *P.* sects. *Zonatae, Poeltiae* Inoue, *Trabeculatae* Inoue, *Fruticosae* Inoue, and *Plagiochila.* The well-supported sister relationship of *P. adiantoides* (Sw.) Lindenb. and *P. cristata* (Sw.) Lindenb. (both in *P.* sect. *Adiantoideae* Lindenb.) within the ITS tree is not reflected in the *rps*4 tree. Here both species are placed in a polytomy with representatives of *P.* sects. *Fuscoluteae* and *Hylacoetes.*

The overall impression is that the ITS tree is better resolved at the sectional level than the *rps*4 tree, whereas the *rps*4 topology exhibits more resolution at some deeper nodes. However, the different trees gave no evidence of incongruence between the nuclear and the chloroplast marker. Consequently, the data sets were combined.

The MP analysis of the combined data sets recovered 314 equally most parsimonious trees (not shown) with a length of 1731 steps, a consistency index (CI) of 0.47, a CI excluding uninformative characters of 0.39, a retention index (RI) of 0.67, and a rescaled consistency index (RC) of 0.32. The strict consensus of these MP Trees (Fig. 3) and the single most likely tree of the ML analysis (Fig. 4; combined data sets) exhibit nearly identical topologies. Several clades of the ML tree, which are well supported by Bayesian analyses, are not or only moderately supported by bootstrap percentage values in the MP tree (*P.* sect. *Durae,* sister relationship of *Durae* and *Glaucescentes,* sister relationship of the *Durae/Glaucescentes* and the *Arrectae/Rutilantes* clades, sister relationship of *P. gymnocalycina* (Lehm. & Lindenb.) Mont. and *P. trichostoma* Gottsche (both in *P.* sect. *Rutilantes*)).

The analyses of the combined data set led to topologies with more robust bootstrap or Bayesian support than the trees derived from the individual data sets. In contrast to the trees derived from the separate data sets, the trees from the combined data sets are well resolved both at deeper nodes and at section level.

Circumscription and range of *Plagiochila* sections as revealed from analyses of the combined data sets (figs. 3, 4)

Plagiochila *sect.* Alternantes

Both ML and MP methods identify *Plagiochila* sect. *Alternantes* as the basalmost clade of *Plagiochila.* In both topologies, the formerly Neotropical *Alternantes* (Heinrichs, 2002) additionally include species from southern South America (*P. chonotica* Taylor) and New Zealand (*P. banksiana* Gottsche and *P. retrospectans* (Spreng.) Lindenb.), leading to a range extension of this group (Fig. 5) and the new synonymy of *P.* sect. *Alternantes* Carl and *P.* sect. *Banksianae* Carl. Morphologically, the *Alternantes* are characterized by their large size, dominating lateral-intercalary branching, and cylindrical to long cylindrical perianths (campanulate in *P. banksiana,* Inoue & Schuster, 1971). Sporophytes are known from the Neotropical *Plagiochila alternans* and *P. ovata* (Müller et al., 1999), as well as from the New Zealand *P. retrospectans* (unpublished, pers. obs.). The sporophytes stand out by their long setae, elongate capsules with cell wall thickenings in all layers of the wall,

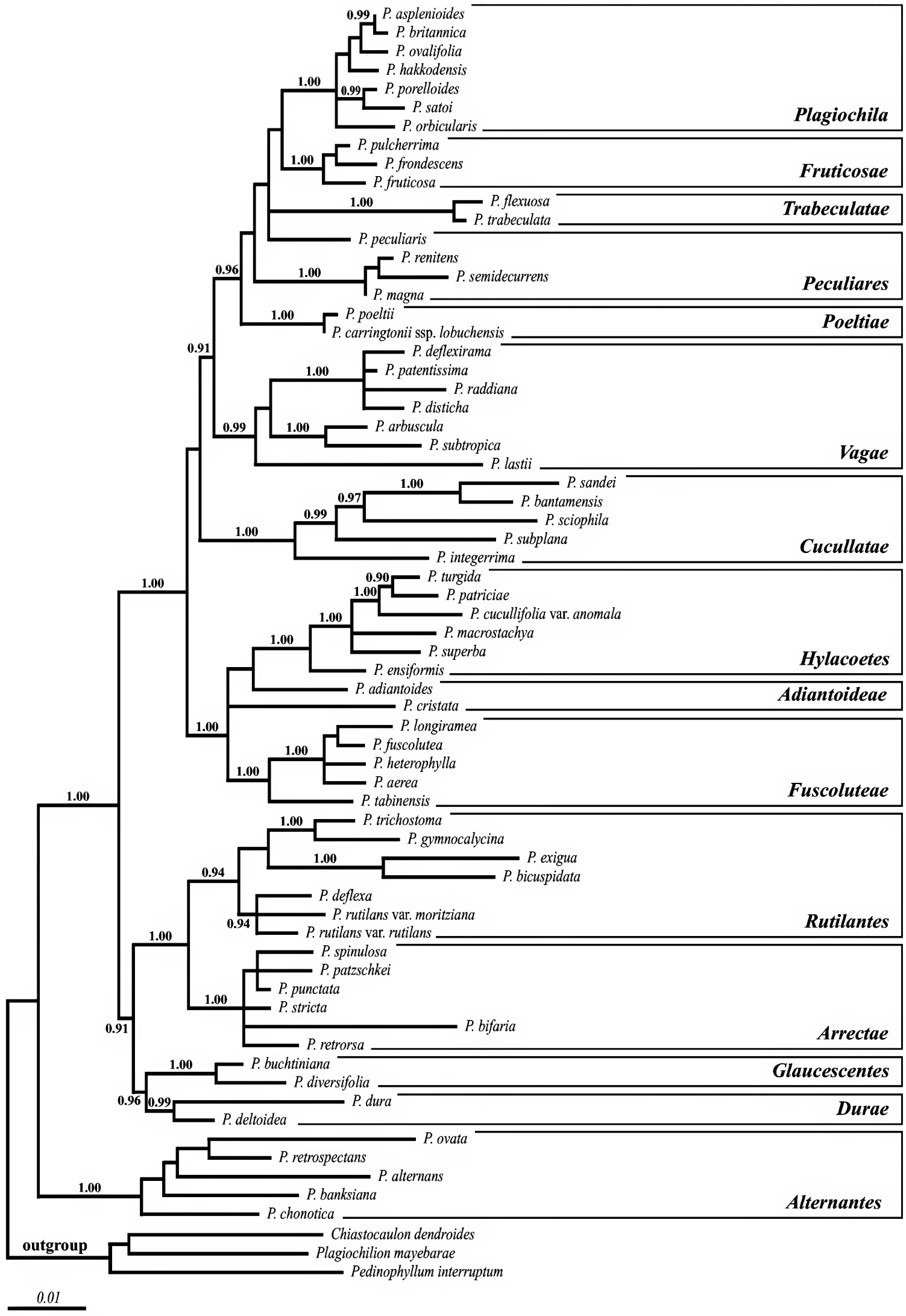

Figure 1. Most likely phylogeny (−ln = 3855.87280) of *Plagiochila* species based on *rps*4 sequence comparisons using 564 aligned positions (primer regions excluded) under the TVM model with estimated gamma shape (G = 0.521), calculated as the best model by Modeltest 3.06. Bayesian support (> 0.90) is indicated above branches.

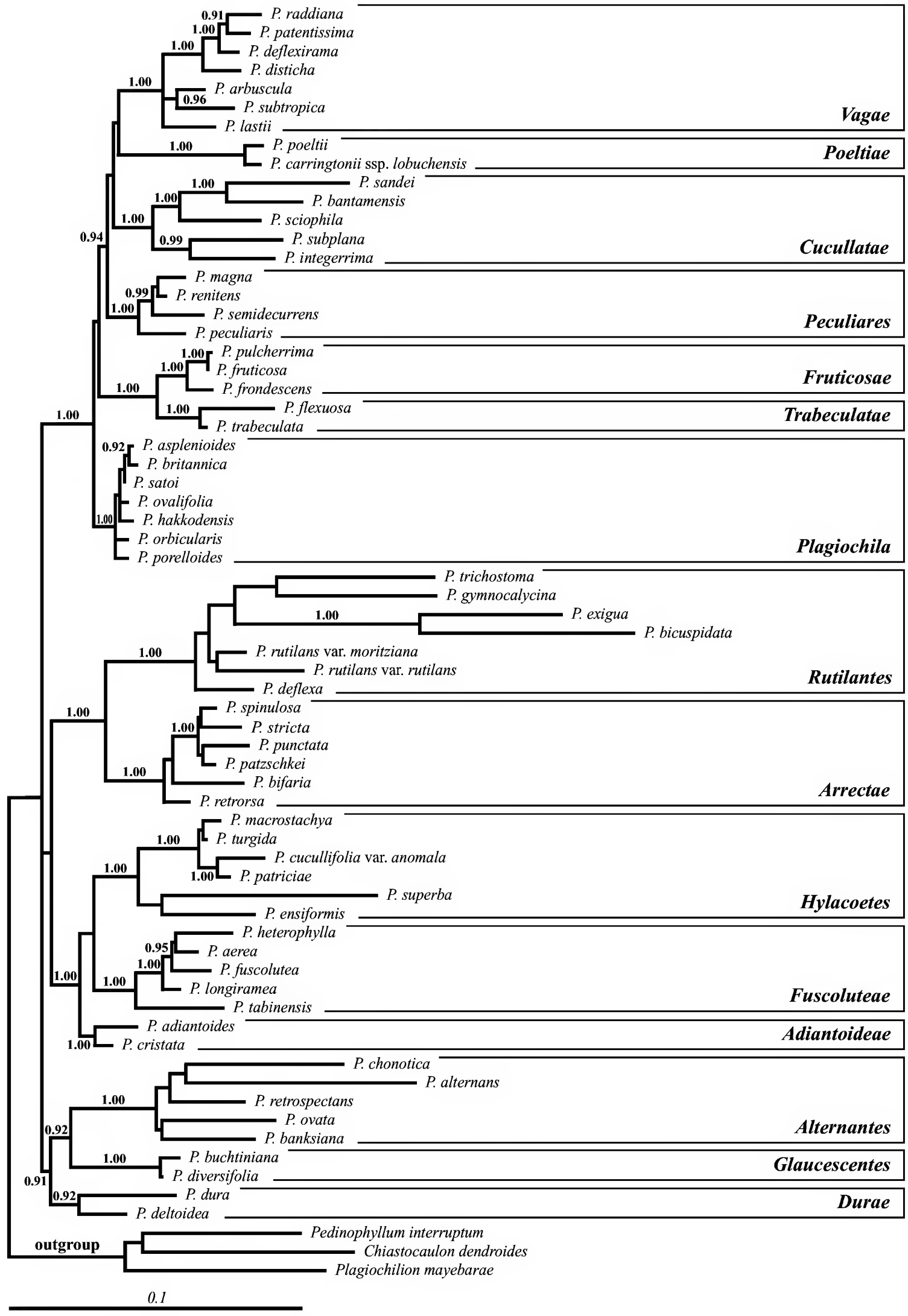

Figure 2. Molecular phylogeny of *Plagiochila* species based on ITS1-5.8S-ITS2 nrDNA sequence comparisons using 666 aligned positions (ambigously aligned regions were excluded). The unique most likely tree (−ln = 7031.23565) resulted from a maximum likelihood analysis, using the TrN model with estimated gamma shape (G = 1.057) and proportion of invariable sites (I = 0.31), calculated as the best model by Modeltest 3.06. Bayesian support (> 0.90) is indicated above branches.

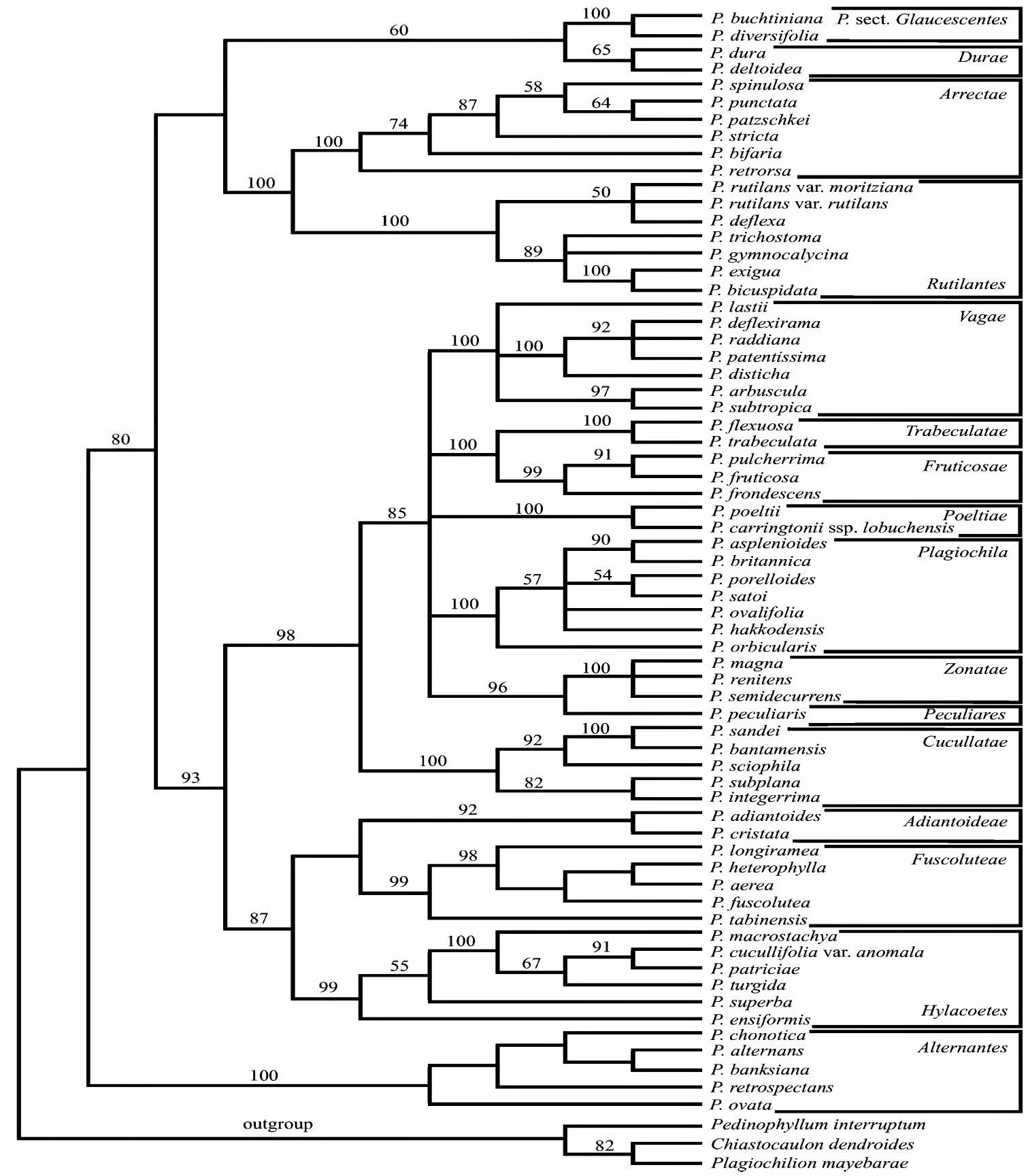

Figure 3. Rooted strict consensus tree of 314 equally parsimonious trees recovered during 1000 random taxon addition heuristic searches of the combined ITS and *rps*4 data sets (1230 characters, 390 parsimony informative). Bootstrap support (> 50%) is indicated above branches.

bispiral, smooth elaters, and small, unicellular spores with verrucate-vermiculate sporoderms.

These characters reoccur in the Holarctic *Plagiochila* sect. *Plagiochila* (e.g., Groth et al., 2003). However, a close relationship of *P.* sects. *Alternantes* and *Plagiochila* as assumed by Müller et al. (1999) is not supported by the molecular topologies, which identify *P.* sect. *Plagiochila* as a derived clade within the genus. No morphological autapomorphisms characterize these sectional clades (compare also Heinrichs, 2002).

Plagiochila *sects.* Arrectae, Durae, Glaucescentes, *and* Rutilantes

The *Alternantes* are placed sister to a large clade that is divided into two lineages of which one is well supported only in the ML analyses. The latter lineage is split into two lines, which each comprise two sectional clades. As in earlier analyses (Groth et. al., 2002, 2003; Heinrichs, 2002; Heinrichs et al., 2002a), *Plagiochila* sect. *Rutilantes* Carl is placed sister to the Neotropical-Atlantic European

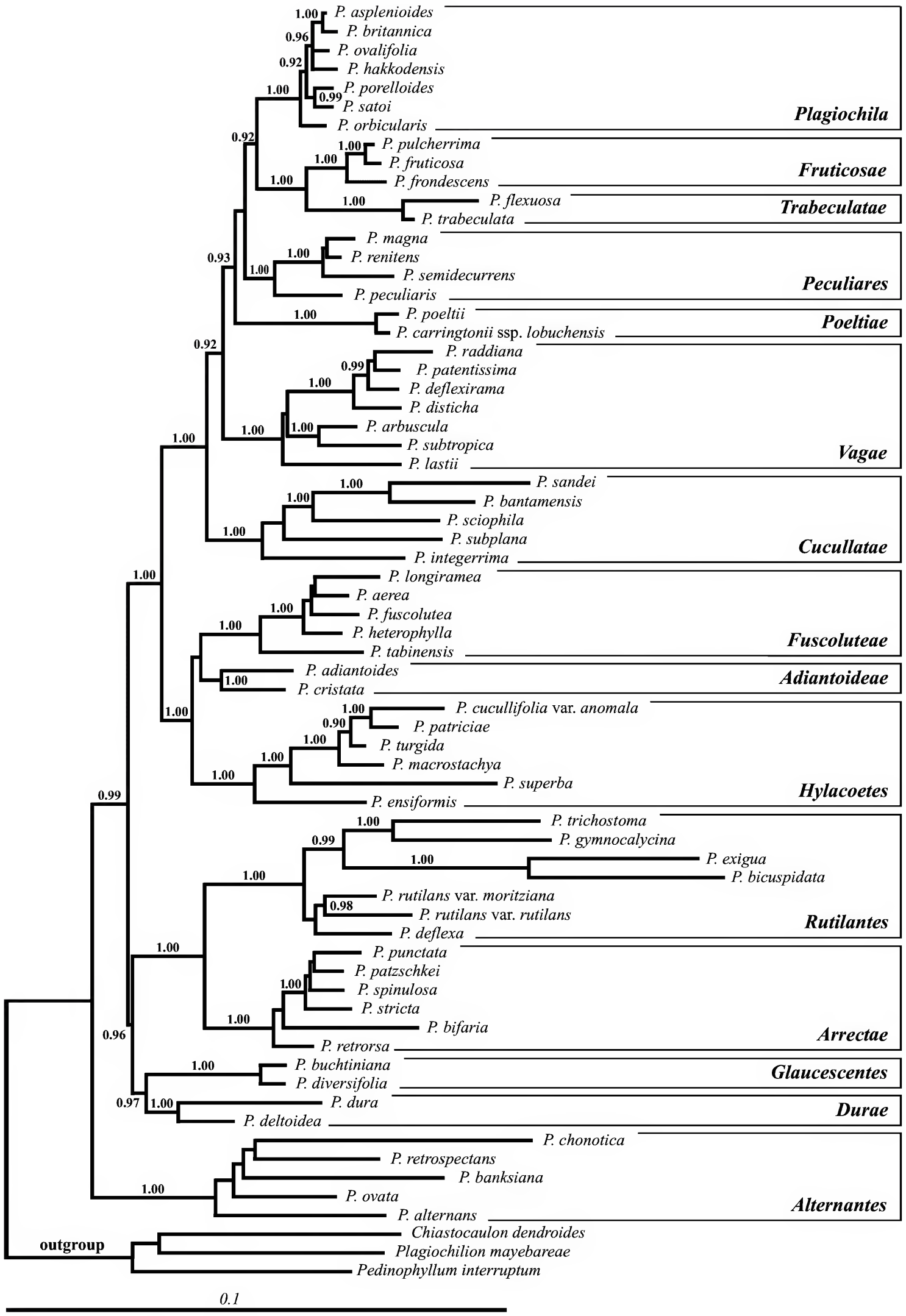

Figure 4. The single most likely tree (−ln = 11,186.41858) resulting from a maximum likelihood analysis of the combined ITS and *rps*4 data sets under the GTR model with estimated gamma shape (G = 0.879) and proportion of invariable sites (I = 0.29), calculated as the best model by Modeltest 3.06. Bayesian support (> 0.90) is indicated above branches.

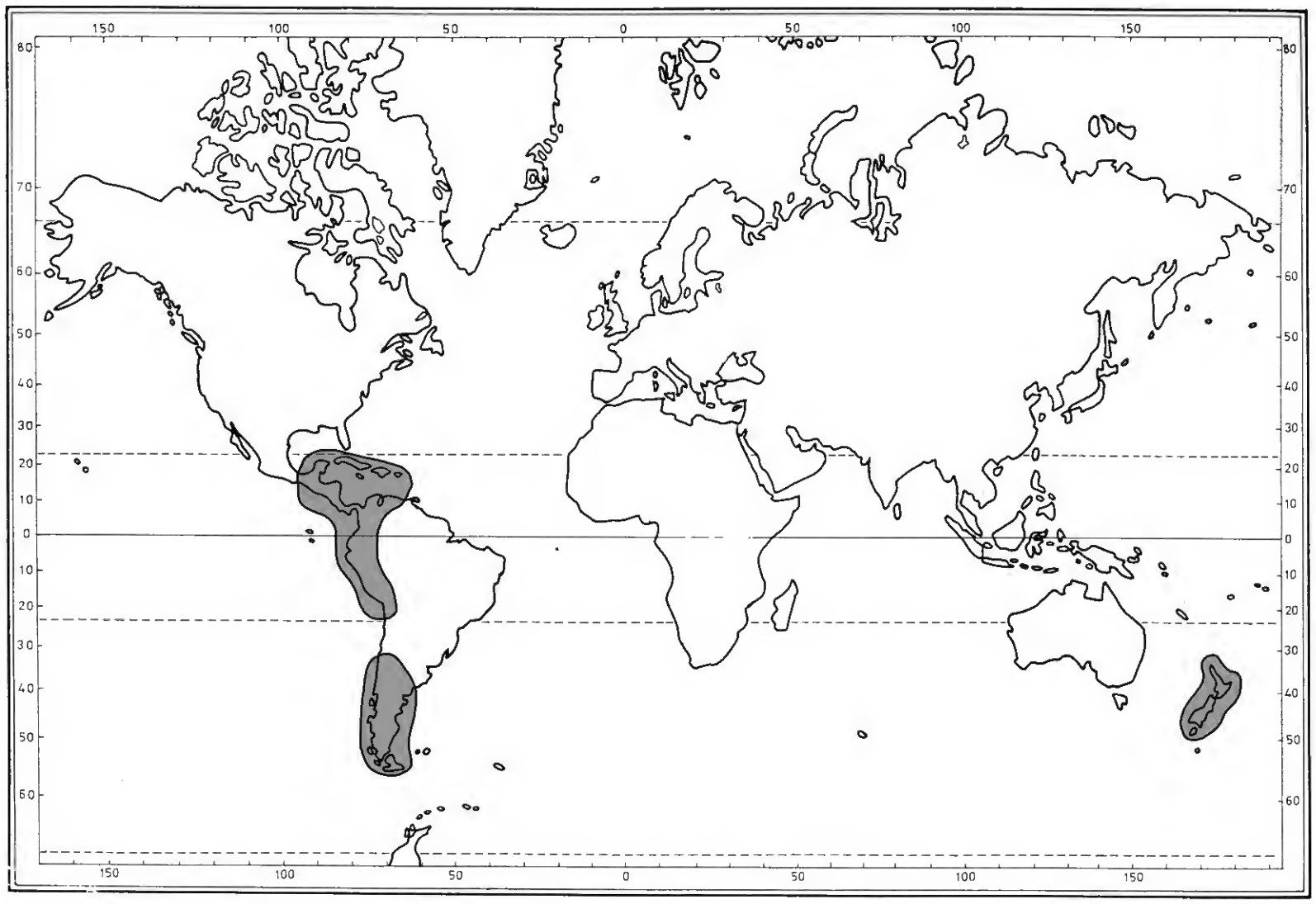

Figure 5. Distribution of *Plagiochila* sect. *Alternantes*.

Plagiochila sect. *Arrectae* Carl. According to the molecular topologies, the formerly purely Neotropical *Rutilantes* occur in southern and tropical South America as well as North America, Europe, Asia, and Africa (Fig. 6).

The *Rutilantes* are morphologically a somewhat heterogeneous group of usually small to medium sized species with predominantly lateral-intercalary branches, simple androecia and a cylindrical perianth that is usually not or only basally covered by the bracts. Some South American representatives stand out by their distinct odor of peppermint (e.g., Heinrichs et al., 2001, 2002a; Groth et al., 2002; Rycroft, 2003).

Plagiochila dura De Not. from southern South America, the type species of the monospecific *P.* sect. *Durae* Carl (Carl, 1931a), is placed sister to *P. deltoidea* Lindenb. (Bayesian support of 1.00). Consequently, the *Deltoideae* are lowered to a synonym of the *Durae*. The *Durae* occur in Patagonia and New Zealand (Fig. 7). Morphologically, both species are only loosely related. *Plagiochila dura* is morphologically more similar to representatives of the *Arrectae* from the Neotropics and Atlantic Europe, e.g., to the type species *P. bifaria* (Heinrichs et al., 1998a, b). As many phenotypes of *P. bifaria* (e.g., Heinrichs et al., 2004a), *P. dura* has numerous lateral-intercalary branches leading to a somewhat pinnate habit (e.g., Carl, 1931a), laterally oppressed leaves with a distinct vitta, capsule walls with thickenings in all layers, baculate, globose spores, and unispiral rough elaters. However, *P. dura* is unique in its equally thickened leaf cell walls and has, contrary to the *Arrectae* species, papillose oil bodies. *Plagiochila deltoidea* has bispiral elaters (Inoue & Schuster, 1971).

The moderately (MP, Fig. 3) to well (ML, Fig. 4) supported sister group of *Plagiochila* sect. *Durae* is the odd Neotropical-Atlantic European *P.* sect. *Glaucescentes* Carl, the only known species group of *Plagiochila* with capsule wall thickenings ± restricted to the epidermal layer (e.g., Heinrichs et al., 2000a). Morphologically, the *Glaucescentes* differ in many respects from the sections resolved here as close relatives (i.e., *Arrectae*, *Durae*, and *Rutilantes*), e.g., by the frequent occurrence of lateral-terminal branches, brown oil bodies, subrotundate perianths, as well as several sporophytic features.

The *Arrectae/Durae/Glaucescentes/Rutilantes* clade is placed sister to a large robust clade, which is split into a line with *Plagiochila* sects. *Adiantoideae*, *Hylacoetes*, and *Fuscoluteae* and a line with *P.* sects. *Cucullatae*, *Fruticosae*, *Trabeculatae*, *Peculiares*, *Plagiochila*, *Poeltiae*, and *Vagae*.

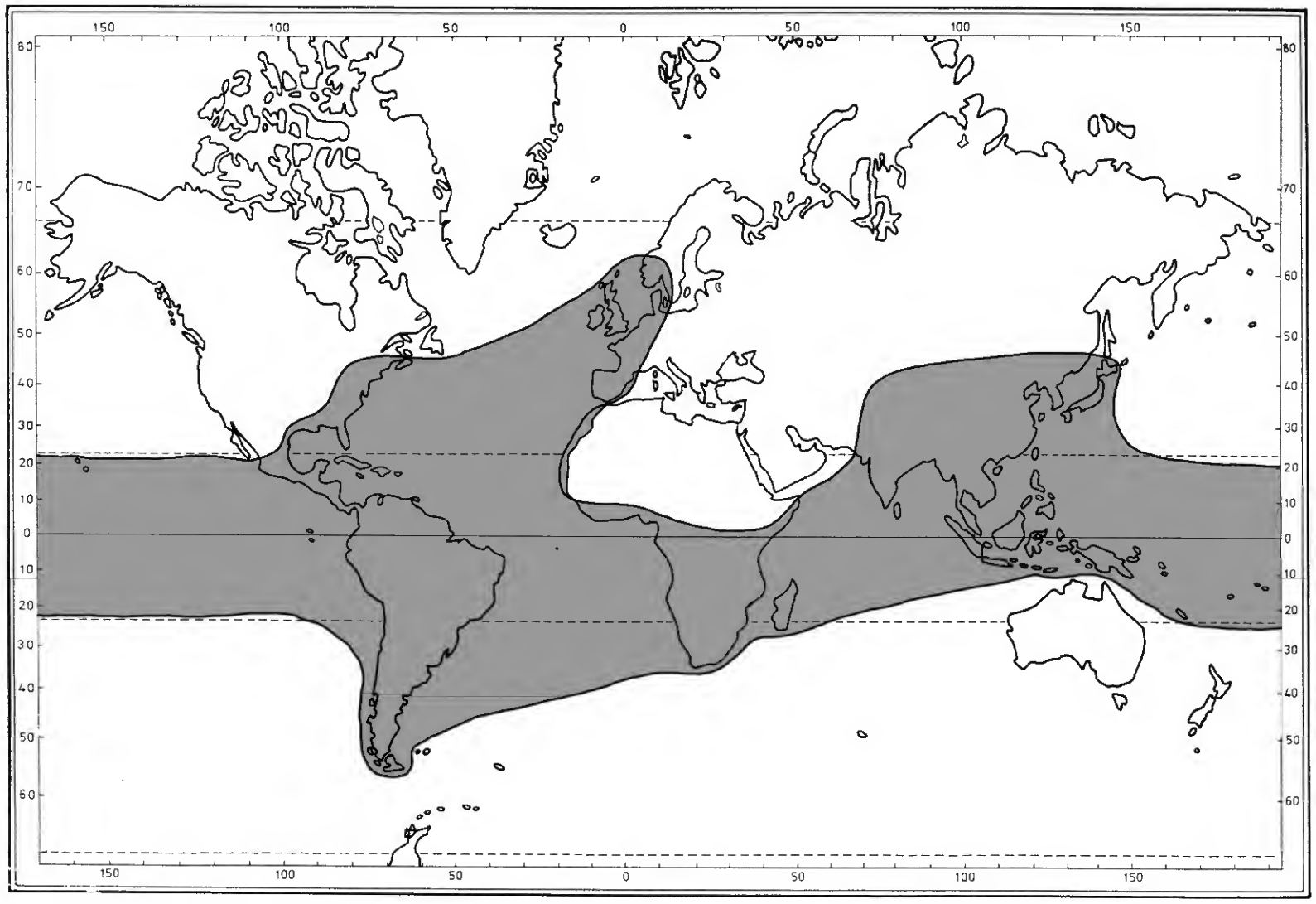

Figure 6. Distribution of *Plagiochila* sect. *Rutilantes*.

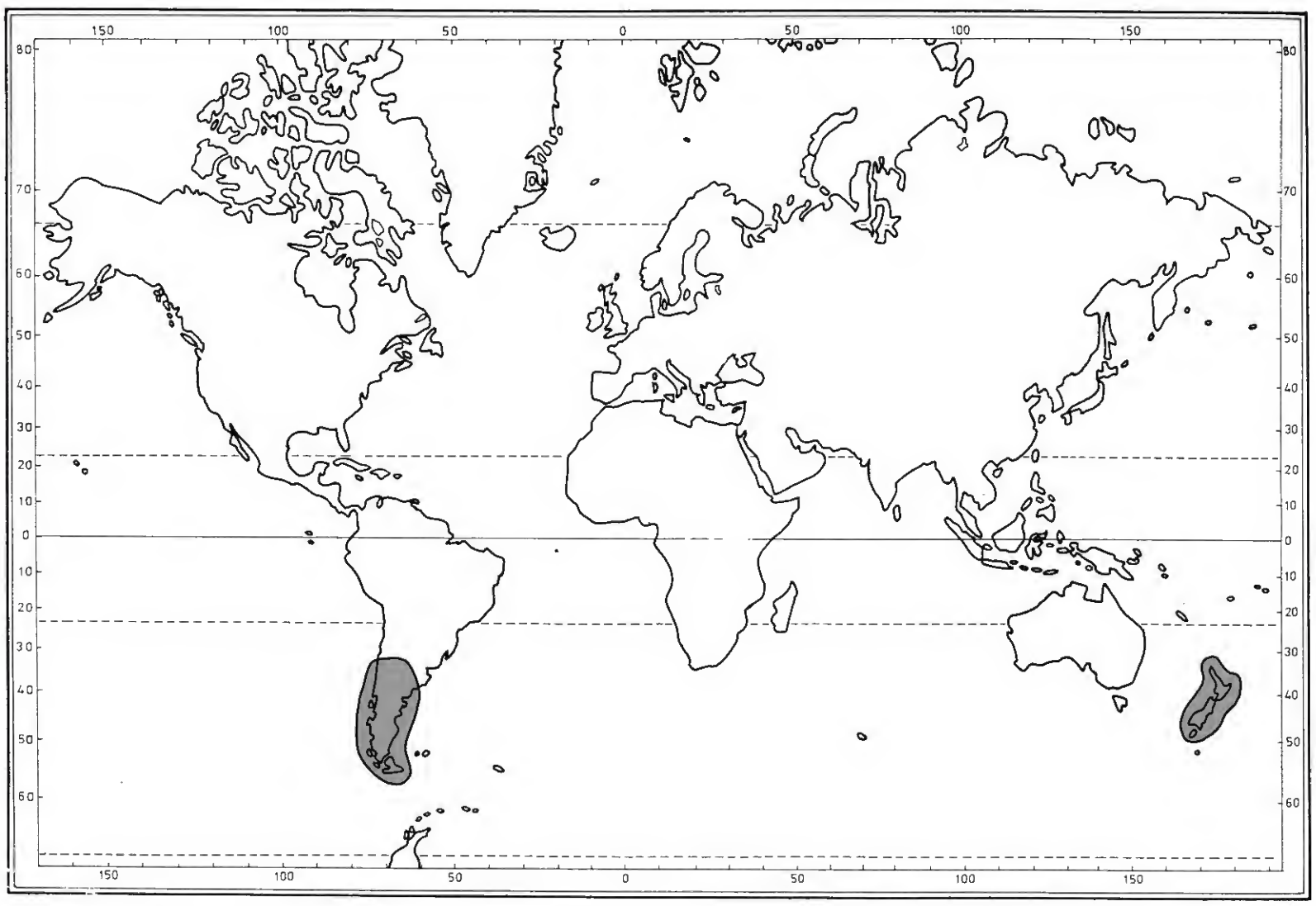

Figure 7. Distribution of *Plagiochila* sect. *Durae*.

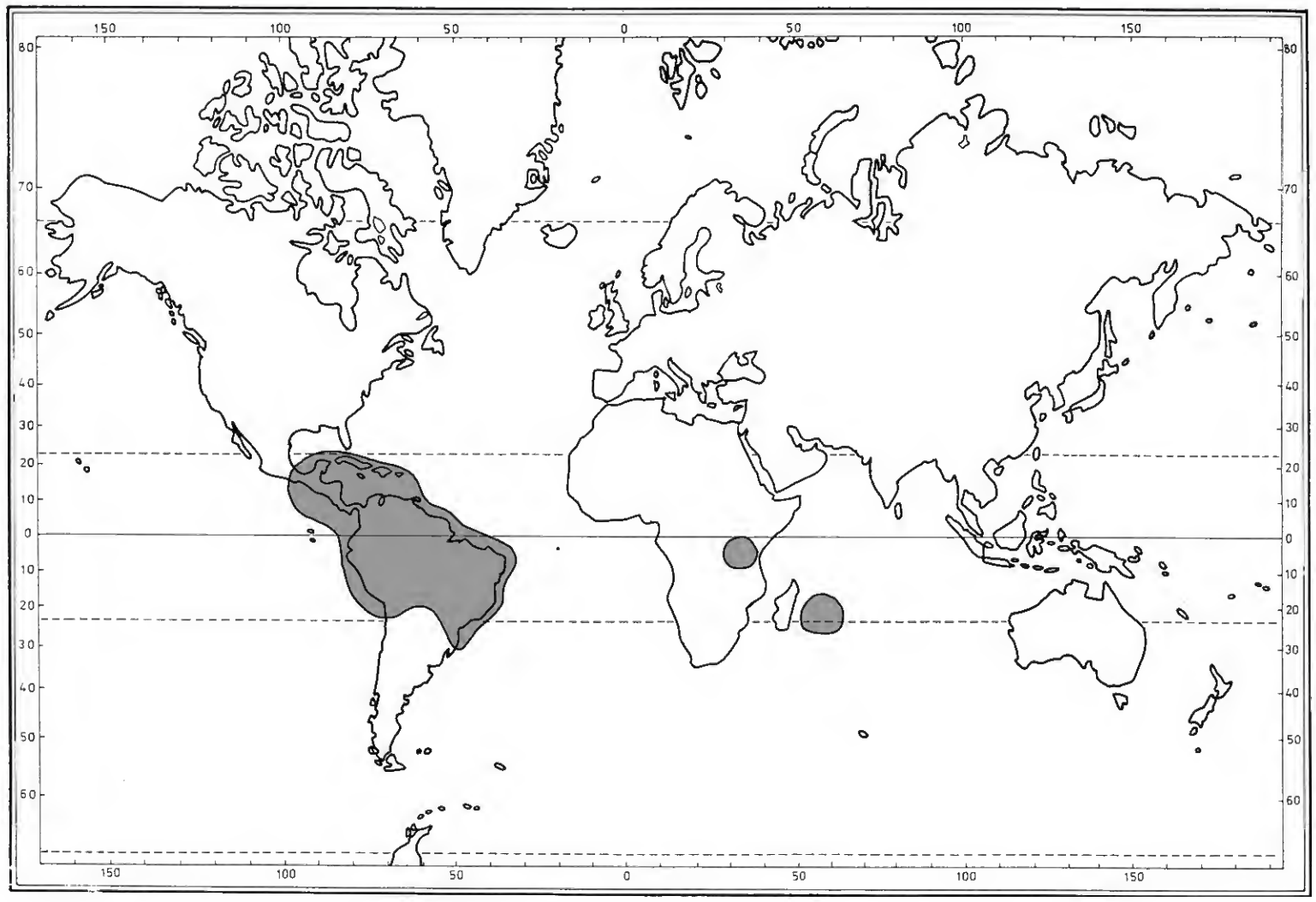

Figure 8. Distribution of *Plagiochila* sect. *Hylacoetes*.

Plagiochila *sects.* Adiantoideae, Fuscoluteae, *and* Hylacoetes

This robust clade is discussed in detail by Heinrichs (2002). The subdivision of the clade into three lineages is supported by morphology. The *Hylacoetes* are characterized by a capsule without cell wall thickenings in the epidermal layer and usually terminal, fan-shaped androecia. Both the *Adiantoideae* and the *Fuscoluteae* have a capsule wall with thickenings in all layers and simple, intercalary androecia. The *Fuscoluteae* differ from the *Adiantoideae* as well as other groups of *Plagiochila* by the occurrence of surface wax (Heinrichs et al., 2000b; Heinrichs, 2002). However, wax was also proven to exist in the outgroup species *Plagiochilion mayebarae* S. Hatt. (Heinrichs, 2000b).

One species of the Neotropical *Hylacoetes*, *Plagiochila boryana* Steph., also occurs in tropical Africa (Heinrichs, 2002; Groth et al., 2003; Fig. 8); one species of the tropical American *Fuscoluteae*, *P. heterophylla* Lehm., is also present in Atlantic Europe (Heinrichs, 2002; Groth et al., 2003; Fig. 9). The small group of the *Adiantoideae* (four species; Heinrichs, 2002) seems to be restricted to tropical America (Fig. 10).

The second large clade is subdivided into several robust sectional lineages with largely unresolved sister relationships (*Plagiochila* sects. *Cucullatae*, *Fruticosae*, *Trabeculatae*, *Peculiares*, *Plagiochila*, *Poeltiae*, and *Vagae*).

Plagiochila *sect.* Vagae

Heinrichs et al. (2002c) lectotypified *Plagiochila* sect. *Vagae* Lindenb. with *Plagiochila patula* (Sw.) Lindenb., leading to a synonymy of this section with the originally Neotropical *P.* sect. *Contiguae* Carl, *P.* sect. *Crispatae* Carl (Carl, 1931a), and other species groups, which are morphologically characterized by the dominance of terminal branching, vegetative distribution by plantlets and propagules on the leaf surface, colorless oil bodies, and a capsule wall with thickenings in all layers (Heinrichs, 2002). *Plagiochila* species from all parts of the tropics and the peripheral regions have been accommodated under the *Vagae* including their synonyms (e.g., Schuster, 1980 (America), Inoue, 1984 (Asia), So, 2000a (Australasia and Pacific)). The molecular topology, which is based on specimens from tropical America (*P. deflexirama* Taylor, *P. disticha* (Lehm. & Lindenb.) Lindenb., *P. patentissima* Lindenb., *P. raddiana* Lindenb.), Africa (*P. lastii* Mitt.), and Asia (*P. arbuscula* (Lehm. & Lindenb.) Lindenb., *P. subtropica* Steph.), supports the previous morphological concepts and confirms the

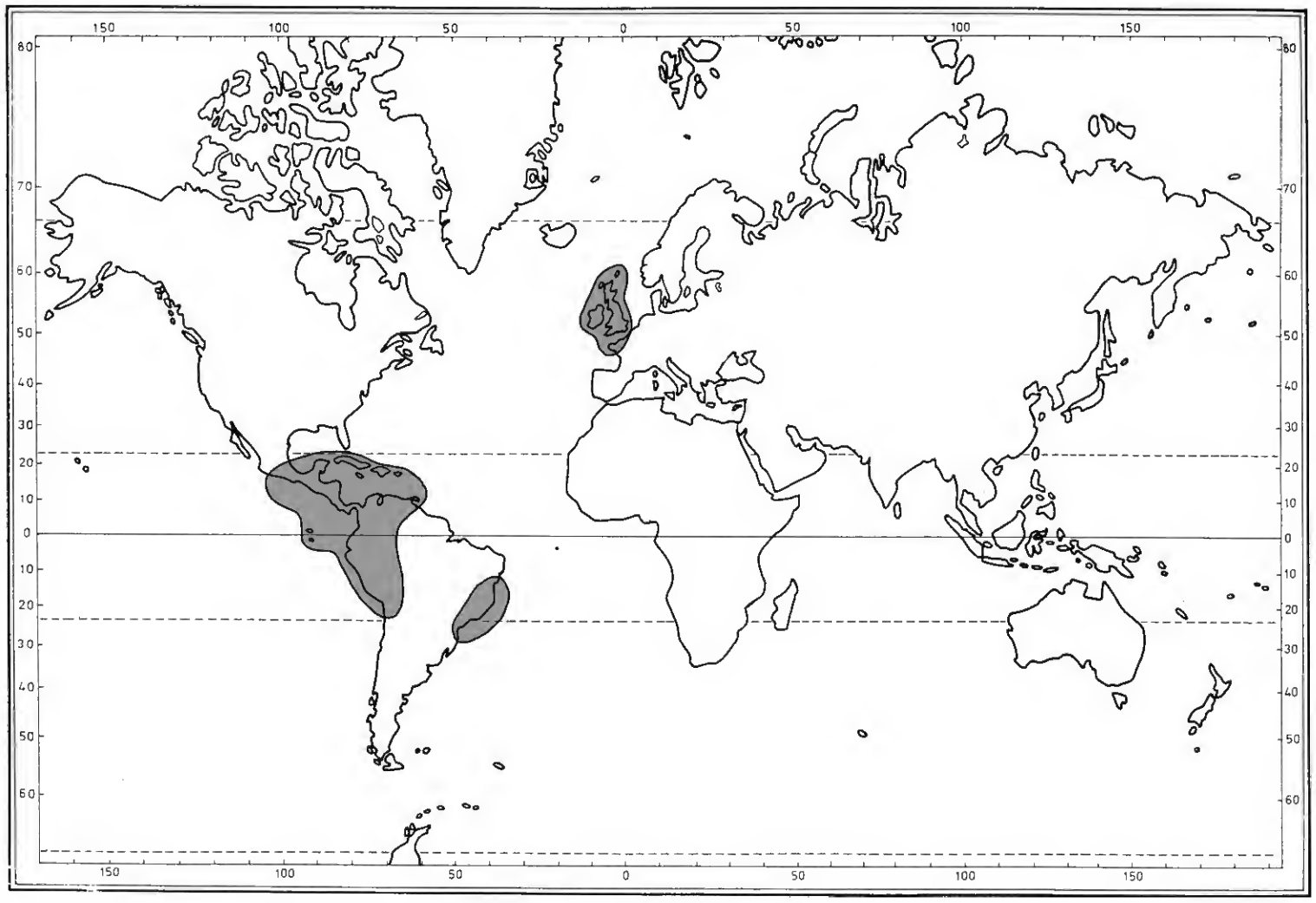

Figure 9. Distribution of *Plagiochila* sect. *Fuscoluteae.*

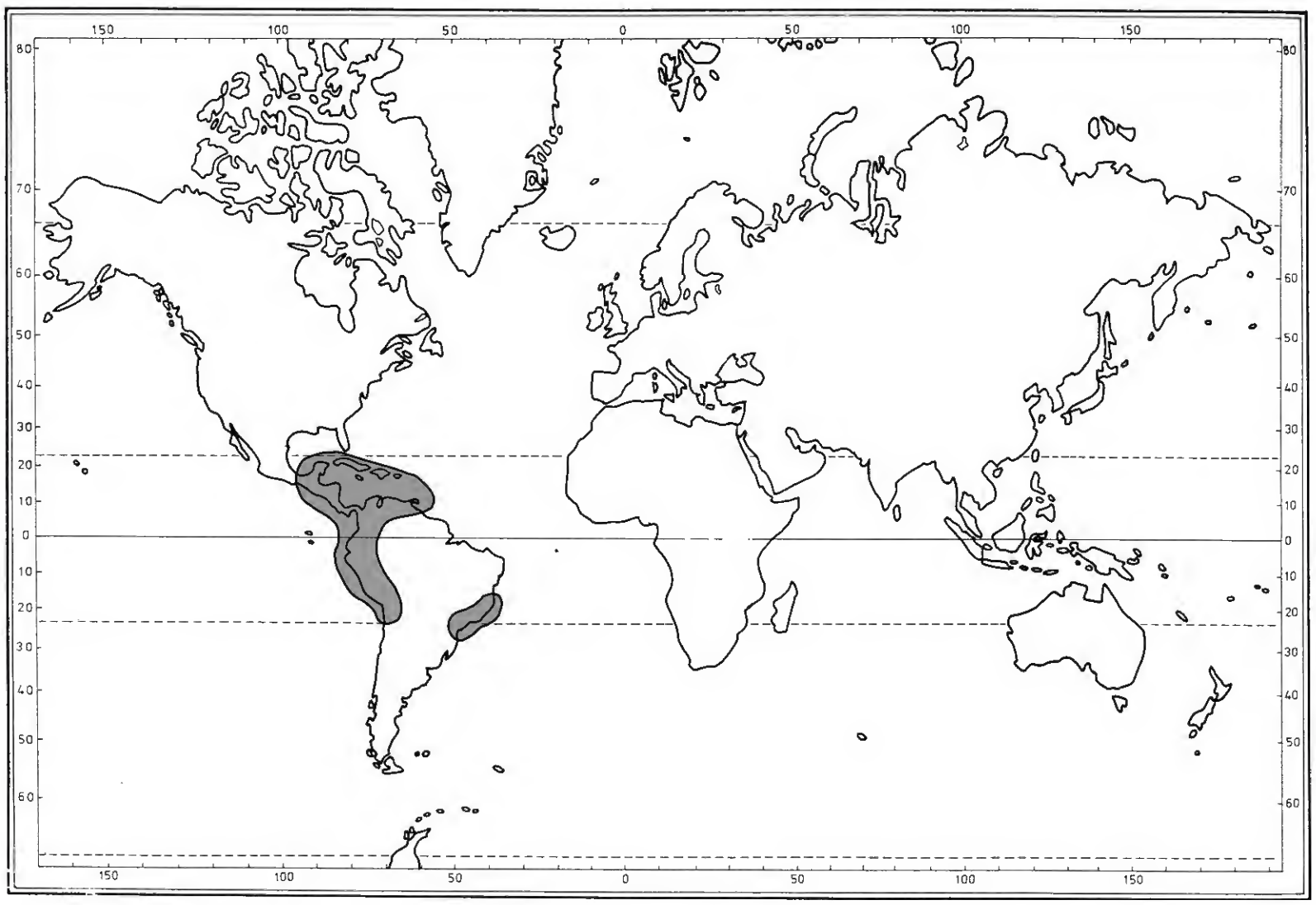

Figure 10. Distribution of *Plagiochila* sect. *Adiantoideae.*

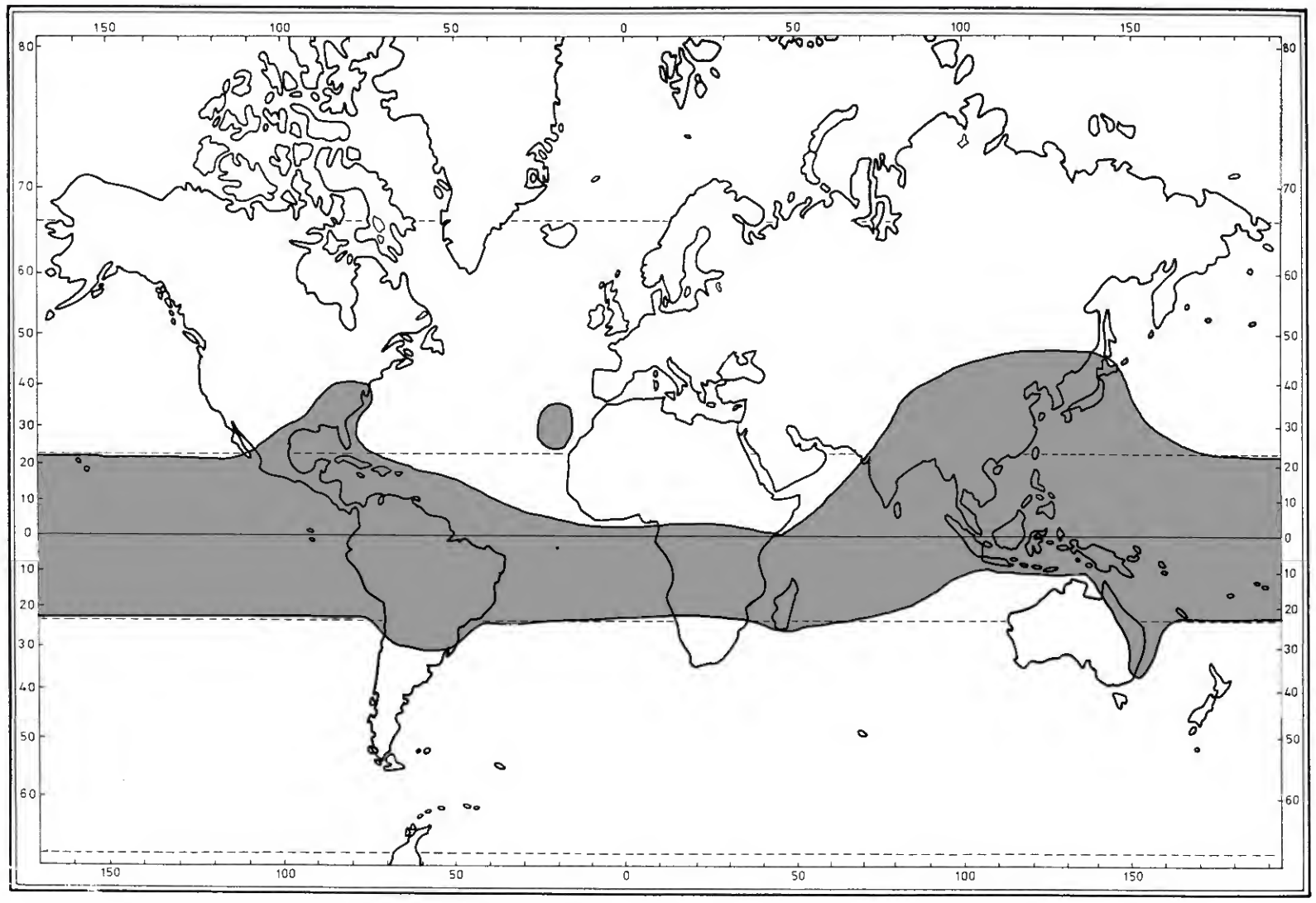

Figure 11. Distribution of *Plagiochila* sect. *Vagae*.

pantropical distribution of the group (Fig. 11). The position of the pantropical *Vagae* in a large robust clade with predominantly Asian and Holarctic *Plagiochila* species suggests that this group descended from Asian ancestors. *Plagiochila* sect. *Parallelae* Carl, set up for Neotropical species with the above characters and oblong leaves, is represented by *P. patentissima*. The species is clearly placed in the well-supported *Vagae* clade. Hence, the section *Parallelae*, which was already tentatively placed in the synonymy of the *Vagae* by Heinrichs (2002), is here formally synonymized. Interestingly, the type of the Asian *P.* sect. *Subtropicae* Carl, *P. subtropica*, is also nested within the *Vagae* clade, leading to a synonymy of both groups. Morphologically, the *Subtropicae* match the features of the *Vagae*; however, vegetative reproduction takes place by means of fragmenting leaves rather than propagules (e.g., Inoue, 1984; So & Grolle, 1999). So and Grolle (1999) point out the occasional occurrence of propagules in several species assigned to the *Subtropicae*. Hence, both groups grade into each other regarding morphology. The similarity of the *Vagae* and the *Subtropicae* is furthermore evident from the difficulties in placing several species that have been moved between both sections (e.g., So & Grolle, 1999). *Plagiochila arbuscula*, placed within the *Tayloriae* Carl by Inoue (1984) and others, is revealed as another member of the *Vagae* clade.

Plagiochila *sect.* Peculiares

Numerous publications are available on the relationships of the Atlantic European/Neotropical *P.* sects. *Arrectae* and the Asian/western North American *Zonatae* (e.g., Schuster, 1980; Grolle & Schumacker, 1982; Rycroft et al., 2001), which both include plants with obliquely oriented leaves with a distinct vitta. However, both groups differ in oil body structure, frequency of paraphyllia, and elater type. Groth et al. (2003) demonstrated that both groups are strictly separated based on ITS sequences. This observation is also confirmed by the analyses of the *rps*4 and the combined data set.

Plagiochila sect. *Peculiares* Schiffn. was set up to accommodate some Asian *Plagiochila* species with flexuose, vermiculate leaf cells (Schiffner, 1900a, 1900b). Inoue (1984) pointed out that the *Peculiares* are otherwise similar to the *Zonatae*. In the molecular trees of the combined data set the type species, *P. peculiaris* Schiffn., is placed at the base of a robust clade with several representatives of the *Zonatae*. This topology justifies a combina-

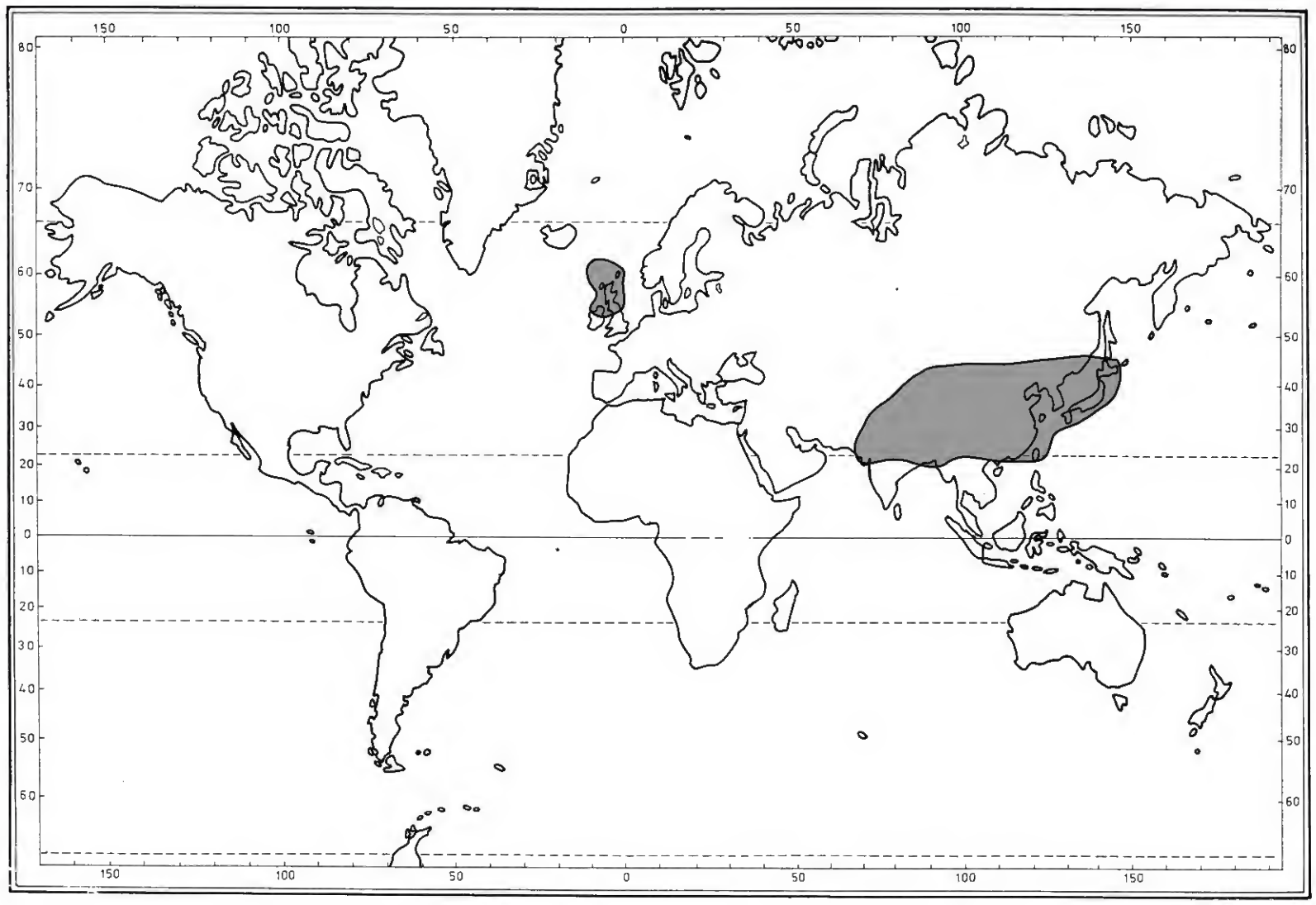

Figure 12. Distribution of *Plagiochila* sect. *Poeltiae.*

tion of both groups under the older name *P.* sect. *Peculiares.*

Plagiochila *sect.* Poeltiae

Plagiochila sect. *Poeltiae* comprises several Asian species, which differ from the broadly circumscribed *Peculiares* mainly by the lack of a vitta. The type species, *P. poeltii* Inoue & Grolle, is placed sister to *P. carringtonii,* the type of the monospecific section *Carringtoniae* Inoue (Inoue, 1965). *Plagiochila carringtonii,* whose disjunct range (Europe/Himalaya) has recently been confirmed by ITS sequence comparisons (Renker et al., 2002), belongs to the most peculiar *Plagiochila* species and has already been placed in five other genera (*Adelanthus, Nardia, Jungermannia, Odontoschisma, Jamesoniella* (Grolle, 1964)). The chloroplast as well as the nuclear marker clearly support the placement of the taxon in *Plagiochila.* Both the ITS and *rps*4 sequences of *P. carringtonii* are largely identical to those of *P. poeltii. Plagiochila carringtonii* stands out by its suborbiculate, more or less entire leaves. In other respects, the species is similar to the *Poeltiae.* As an outcome of the molecular study, both sections are combined, leading to a range extension of the Asian *Poeltiae* to Europe (Fig. 12).

Plagiochila *sect.* Plagiochila

The type section of *Plagiochila* is made up by several Holarctic species close to the generic type *P. asplenioides* (L.) Dumort. Genetic distances between the species of section *Plagiochila* are low and reach levels that are otherwise accepted as infraspecific variation within *Plagiochila* (e.g., Rycroft et al., 2002; Groth et al., 2003; Heinrichs et al., 2004a, b). The low genetic variation of the species is reflected in difficulties in establishing clear morphological boundaries between the taxa (compare, e.g., Schuster, 1980; Paton, 1999; Damsholt, 2002). Center of diversity of *P.* sect. *Plagiochila* is the Holarctis; some species, however, reach the Paleotropics (Fig. 13). The numerous similarities of representatives of *P.* sects. *Plagiochila* and *Alternantes* (see relevant section) are obviously the result of parallel evolution.

Plagiochila *sects.* Fruticosae *and* Trabeculatae

Plagiochila sect. *Fruticosae* Inoue includes species with dendroid habit and mainly Asian distribution (e.g., Inoue, 1984). Placement of *Chiastocaulon dendroides* (Nees) Carl within this group (e.g., So 2001b) was not confirmed by an earlier molecular investigation (Groth & Heinrichs, 2003),

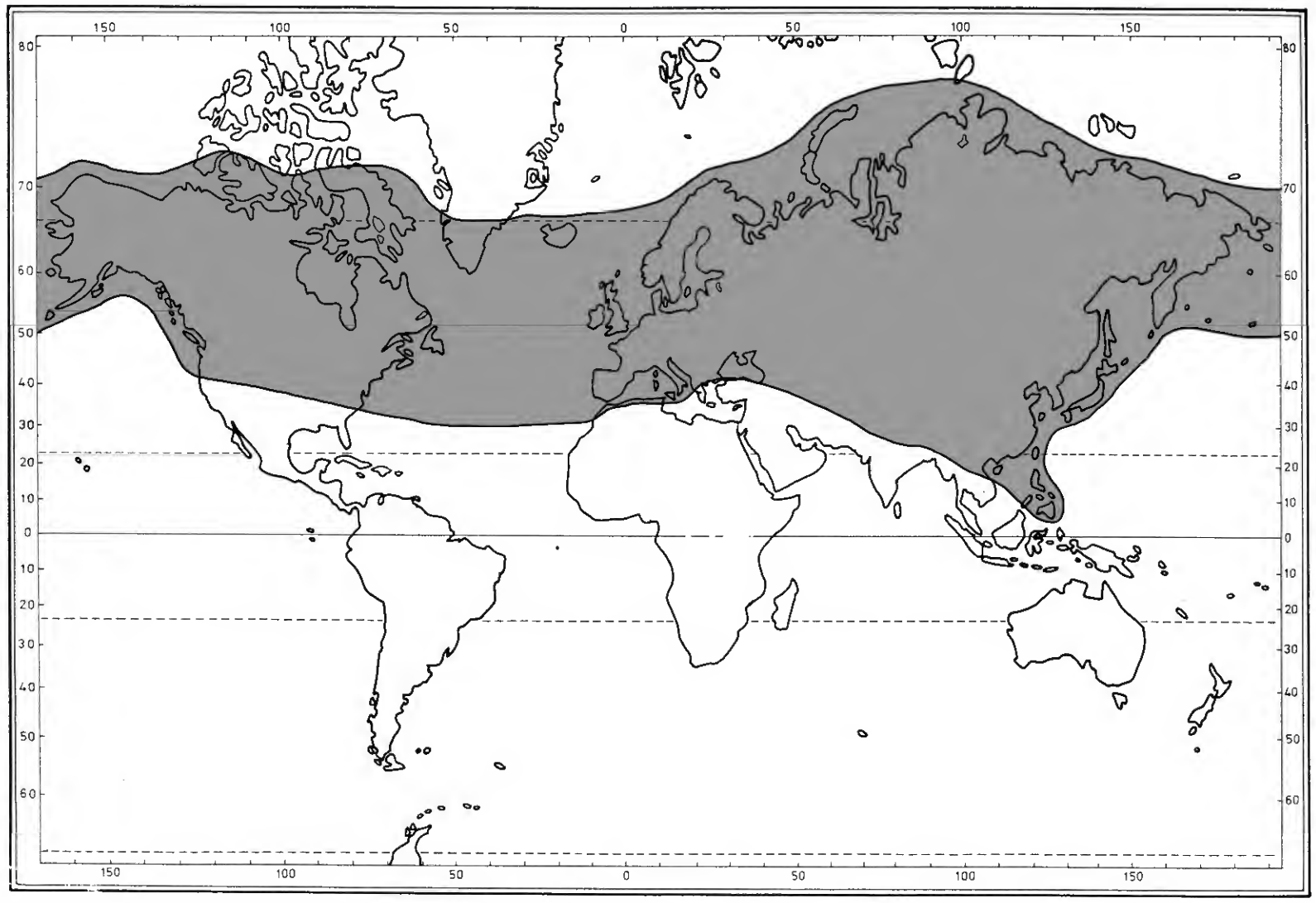

Figure 13. Distribution of *Plagiochila* sect. *Plagiochila*.

which allowed us to allocate the species as an outgroup of *Plagiochila* in this study. Morphologically, *Chiastocaulon* differs from the representatives of *P.* sect. *Fruticosae* by the frequent occurrence of ventral-intercalary branches (e.g., Carl, 1931b). Within the outgroup, *Chiastocaulon* is placed sister to *Plagiochilion* (Figs. 3, 4), albeit with robust support only in the MP tree (Fig. 3). Both genera possess ventral intercalary branches; however, *Chiastocaulon* has alternating leaves, whereas the leaves of *Plagiochilion* are subopposite.

Plagiochila sect. *Fruticosae* is placed in a well-supported sister relationship with *P.* sect. *Trabeculatae* Inoue, an Asian species group made up by medium-sized *Plagiochila* species with oblong leaves and lateral intercalary as well as terminal branches but without dendroid habit. Several authors (e.g., Inoue, 1984; So, 2001c) have lumped the *Trabeculatae* and the Neotropical *P.* sect. *Cobanae* Carl together. However, the morphology of the Ecuadorian *P. cobana* Steph. has never been studied properly. Judging from the scanty type fragment in the Stephani herbarium in G, *P. cobana* could be related either to *P.* sect. *Rutilantes* or *P.* sect. *Vagae*, which form separate clades in the molecular topologies. Accordingly, the name *P.* sect. *Trabeculatae* is kept in this study.

Plagiochila *sect.* Cucullatae

Plagiochila sect. *Cucullatae* represents a robust lineage at the base of the large *Fruticosae/Trabeculatae/Peculiares/Plagiochila/Poeltiae/Vagae* clade. However, the sister relationship is well supported only in the MP analyses (Fig. 3). The *Cucullatae* were initially set up for a group of tropical Asian *Plagiochila* species with a distinct pseudocucullum (e.g., Schiffner, 1900a, b; Inoue, 1984). Except for the type species *Plagiochila sandei* Sande Lac. (Grolle, 1976), the *Cucullatae* are provided with large underleaves. In other respects, the group is essentially similar to the Neotropical/African *P.* sect. *Hylacoetes*. Both groups produce a capsule with a thin-walled epidermal layer. As in earlier molecular analyses (e.g., Heinrichs, 2002; Groth et al., 2003) *Cucullatae* and *Hylacoetes* are strictly separated. The numerous morphological similarities must be regarded as being homoplastic.

Based on ITS sequence analyses, Groth et al. (2003) recovered the Neotropical *P. subplana* Lindenb. and the African *P. integerrima* Steph. as further representatives of the *Cucullatae*. The sporophyte of *P. integerrima* is still not known, whereas *P. subplana* has a capsule wall similar to that of other *Cucullatae* representatives (Heinrichs et al.,

1999). A pseudocucullum is lacking in both species. Adding *rps*4 sequences to the ITS alignment leads to the same position of *P. integerrima* and *P. subplana* as in the ITS tree of Groth et al. (2003).

Subdivision of Plagiochila *above section level*

Several robust nodes were discovered above section level. Two large clades are well supported both in ML and MP analyses: the Neotropical *Adianto-ideae/Fuscoluteae/Hylacoetes* clade and the mainly Holarctic/Asian *Cucullatae/Fruticosae/Peculiares/Plagiochila/Poeltiae/Vagae* clade with two pantropical elements, the *Vagae* and the *Cucullatae*. A third clade comprising the *Arrectae, Durae, Glaucescentes,* and the *Rutilantes* is only well supported in ML analyses and covers nearly the complete range of *Plagiochila* with the exception of the northernmost parts of the Holarctics. No distinct morphology supports these clades. Previous hierarchical subdivisions of *Plagiochila* are not reflected in the molecular topologies. Spruce's Ramiflorae and Cauliflorae do not form a monophyletic lineage. The majority of the Ramiflorae is concentrated in the *Vagae*; however, the *Glaucescentes* clearly form a separate lineage.

The types of the *Plagiochila* subgenera *Metaplagiochila* and *Paraplagiochila, P. sandei* and *P. peculiaris* (Inoue, 1984), are nested within the generitype clade and are treated as synonyms of subgenus *Plagiochila*. The remaining monospecific *Plagiochila* subgen. *Pleurochila* could not be evaluated due to lack of fresh plant material.

NEW SYNONYMS

Plagiochila sect. **Alternantes** Carl, Ann. Bryol. (suppl. 2): 75. 1931. TYPE: *Plagiochila alternans* Lindenb. & Gottsche.

Plagiochila sect. *Banksianae* Carl, Ann. Bryol. (suppl. 2): 138. 1931. Syn. nov. TYPE: *Plagiochila banksiana* Gottsche.

Plagiochila sect. **Durae** Carl, Ann. Bryol. (suppl. 2): 123. 1931. TYPE: *Plagiochila dura* De Not.

Plagiochila sect. *Deltoideae* Hodgs., Trans. Roy. Soc. N.Z. 73: 271. 1944. Syn. nov. TYPE: *Plagiochila deltoidea* Lindenb.

Plagiochila sect. **Peculiares** Schiffn., Denkschr. Kaiserl. Akad. Wiss., Math.-Naturwiss. Kl. Wien 70: 160. 1900. TYPE: *Plagiochila peculiaris* Schiffn.

Plagiochila sect. *Zonatae* Carl, Ann. Bryol. (suppl. 2): 121, 123. 1931. Syn. nov. TYPE: *Plagiochila zonata* Steph.

Plagiochila sect. **Poeltiae** Inoue, Bull. Natl. Sci. Mus., Tokyo 8: 394. 1965. TYPE: *Plagiochila poeltii* Inoue & Grolle

Plagiochila sect. *Carringtoniae* Inoue, Bull. Natl. Sci. Mus., Tokyo 8: 377. 1965. Syn. nov. TYPE: *Plagiochila carringtonii* (Balfour) Grolle

Plagiochila sect. **Vagae** Lindenb., Spec. Hepat. (fasc. 5): XV. 1843. Lectotype, designated by Heinrichs et al. (2002c: 7), *Plagiochila patula* (Sw.) Lindenb.

Plagiochila sect. *Parallelae* Carl, Ann. Bryol. (suppl. 2): 1931. Syn. nov. TYPE: *Plagiochila parallela* Steph.

Plagiochila sect. *Subtropicae* Carl, Ann. Bryol. (suppl. 2): 81. 1931. Syn. nov. TYPE: *Plagiochila subtropica* Steph.

Plagiochila subgen. **Plagiochila**

Plagiochila subgen. *Paraplagiochila* Inoue, The genus *Plagiochila* (Dum.) Dum. in Southeast Asia: 44. 1984. Syn. nov. TYPE: *Plagiochila peculiaris* Schiffn.

General Discussion

The nuclear ribosomal ITS region (ITS1-5.8S-ITS2) is still the most frequently utilized marker to infer phylogenetic relationships from species to family level (e.g., Baldwin et al., 1995; Soltis & Soltis, 1998; Hershkowitz et al., 1999). However, the usefulness of the ITS marker for phylogenetic reconstructions within bryophytes must be tested as the case arises because variation can be so high that successful alignment is not possible even at species level (Shaw et al., 2002).

Although the Plagiochilaceae are at least 50 million years old (Grolle & Heinrichs, 2003), large parts of both ITS1 and ITS2 sequences can be aligned successfully and lead to well-resolved topologies. Within Lejeuneaceae, ITS1 is more variable than ITS2 and only a limited number of ITS1 positions can be used for phylogenetic analyses (Groth-Malonek et al., 2004).

The chloroplast encoded *rps*4 gene is widely used to infer the phylogeny of mosses (e.g., Goffinet et al., 2001). Within leafy liverworts it has previously been utilized only in the Plagiochilaceae (Groth & Heinrichs, 2003). Although somewhat less variable than ITS, *rps*4 is informative at species level and largely reflects the ITS topologies. Analogue variation of ITS and *rps*4 is not a general phenomenon. Within *Sphagnum rps*4 is only slightly variable, whereas ITS alignments lead to well-resolved trees (Shaw, 2000).

For a long time, bryophyte taxonomy has been based solely on morphology. Molecular analyses often offer new insights into the phylogeny of this

important lineage and allow the evaluation of previous morphology-based hypotheses. Only a limited number of molecular investigations of larger families or genera of bryophytes is available to date (e.g., Amblystegiaceae, Vanderpoorten et al., 2002; *Asterella,* Long et al., 2000; Bryaceae, Cox & Hedderson, 2003; Pedersen et al., 2003; Dicranaceae, La Farge et al., 2002; Fontinalaceae, Shaw & Allen, 2000; *Plagiochila,* Heinrichs, 2002; Groth et al., 2003; Pottiaceae, Werner et al., 2002; *Sphagnum,* Shaw, 2000). The overall impression derived from these studies is that morphological data sets of bryophytes are highly homoplastic and that numerous taxonomic changes are necessary to establish natural subdivisions. The taxonomic value of morphological characters used so far for the circumscription of supraspecific units appears in a new light when molecular data are taken into account. Cox and Hedderson (2003) disprove the dictum that mosses with similar peristomes must be placed together. Vanderpoorten et al. (2002) pointed out that sporophytic specializations are among the most homoplastic within the Amblystegiaceae. On the other hand, sporophytic features within *Plagiochila* sections as derived from molecular topologies are quite stable; however, similar sporophyte types often occur in several sectional clades. In *Plagiochila,* the *Alternantes* and section *Plagiochila,* the *Adiantoideae* and the *Fuscoluteae,* as well as the *Hylacoetes* and the *Cucullatae,* share their sporophytic characters. Spores and elaters often exhibit more variation at section level than capsule wall features. *Plagiochila* sect. *Vagae* (e.g., Inoue, 1984; Heinrichs & Gradstein, 2000; Heinrichs, 2002) comprises species with unispiral as well as with bispiral elaters; the surface may be smooth or rough. Spores within the *Vagae* may be globose or cylindrical and are unicellular or build by up to ca. seven cells.

Most gametophytical characters of *Plagiochila* are even more homoplastic than features derived from the sporophyte. Leaf shape—utilized as the main supraspecific character by Stephani (1901–1906) and Dugas (1929)—as well as leaf dentation have nearly no taxonomic value above species level. Paraphyllia are present in species belonging to very different sections. They are known from, for example, representatives of the *Fruticosae, Fuscoluteae, Peculiares,* and *Vagae.* A similar situation exists concerning the variation of the underleaves (e.g., Heinrichs, 2002).

Other characters must be reappraised for each section. Oil body variation is low within the *Glaucescentes* and the *Rutilantes* in their current circumscription, whereas three *Adiantoideae* species all have their own oil body type (Heinrichs, 2002). At least vigorous plants of all *Vagae* representatives identified so far are characterized by dominating terminal branching. In contrast, *Plagiochila* sect. *Fuscoluteae* or section *Rutilantes* incorporate both species with exclusively intercalary branching and species with both types.

Only the minority of *Plagiochila* sections is supported by morphological autapomorphies. Wax is present in all *Fuscoluteae* representatives and thin-walled inner capsule wall layers as well as partly echinate sporoderms are restricted to the *Glaucescentes.* However, although lacking morphological autapomorphies, many sectional *Plagiochila* clades can be distinguished by a combination of several morphological features (cf. e.g., Heinrichs, 2002).

The effects of the molecular investigations on the sectional subdivision of *Plagiochila* are clearly positive with respect to the assignment of species. Although many supraspecific groups of the old authors were simply designed as pigeonholes for a certain morphotype, the high infraspecific variation and the poorness in morphological characters hampered the classification of many species. Accordingly, a considerable number of recent morphology papers (e.g., Heinrichs & Gradstein, 1999, 2000; So, 2001c) still focus on the sectional placement of species and sectional boundaries. Studying the 83 pages of Schuster's (1980) treatment of the *Plagiochila* sections *Contiguae, Crispatae, Hypnoides,* and *Parallelae* is unconvincing with regard to the classification of species not treated in this flora. However, the molecular investigations reveal the above and several other sectional taxa as members of a very natural group, which can now easily be identified by the frequent occurrence of terminal branches, colorless oil bodies, a capsule wall with thickenings in all layers, and, in many cases, the occurrence of propagules and plantlets on the leaves of its members.

About 100 *Plagiochila* binomials or ca. 20%–25% of the morphospecies worldwide have so far been included in the molecular analyses. Although taxa from the southernmost part of the genus's range are still inadequately represented, some statements are already possible.

Our preliminary results suggest that not more than 25% of the more than 100 supraspecific taxa of *Plagiochila* will be reflected in molecular topologies. The molecular investigations may generally lead to a reduction of *Plagiochila* sections similar to the results of Shaw (2000) with regard to *Sphagnum.* As revealed from our molecular trees, some sectional clades are pantropical in distribution. Some clades are ± restricted to the Holarctics or

to tropical America and Europe. Other clades cover nearly the complete generic range or are restricted to South America and Australasia. Geographic concepts as applied to by Carl (1931a) are not supported by the molecular analyses: the majority of *Plagiochila* sections as derived from the molecular trees span several floristic kingdoms. However, due to the frequency of homoplasy it is not possible to identify species groups by morphology alone. Integration of molecular data from a larger number of species is required before a natural classification can be approached in *Plagiochila*. The present molecular topologies lead to a synthesis of Carl's (1931a) and Lindenberg's (1839–1844) concepts. They demonstrate that the geographic origin of a species enhances the likelihood of affiliation to a certain clade. According to the molecular topologies, plants from the Neotropics with intercalary branching, laterally oppressed leaves with a vitta, simple intercalary androecia, perianths covered by the bracts at least in the basal half, and rough unispiral elaters may belong to the *Plagiochila* sect. *Arrectae*. Species with the same character set from southern South America may belong to the section *Durae*.

Molecular data clearly demonstrate that the *Plagiochila* taxa from Atlantic Europe belong to Neotropical or Pantropical sections or to sections from Asia. Contrary to earlier belief (e.g., Herzog, 1945; Jones & Rose, 1975), these taxa do not represent endemics of Europe but belong to far more widespread species (overview in Groth et al., 2003). The European stands represent only the edge of wider ranges and seem to be the result of rather recent spore dispersal events (e.g., Heinrichs et al., 2004a). This situation is not unexpected with regard to the climatic changes in Europe during the last 100,000 years (e.g., Kaiser, 1967). The cold and dry climate of the ice ages including the interstadials surely prevented the presence of species adapted to humid and warm conditions. Evidence for the conspecifity of Atlantic European endemic liverwort taxa with tropical binomials has recently also been provided for, e.g., *Radula voluta* Taylor (Reiner-Drehwald, 1994) and *Herbertus borealis* Crundw. (Hodgetts, 2003).

The most basal clade of *Plagiochila*, representing *P.* sect. *Alternantes*, is likely the result of an Gondwanan vicariance similar to that of *Monoclea* (Gradstein et al., 1992; Meißner et al., 1998). The unexceptionally long branches of all members of the *Alternantes* clade point to a long time of reproductive isolation. Long isolation periods are not a general phenomenon of bryophytes with a Gondwanan distribution. Fast-evolving molecular markers of *Leptotheca gaudichaudii* Schwaegr. (McDaniel & Shaw, 2003), *Lopidium concinnum* (Hook.) Wilson (Frey et al., 1999) or *Hypopterygium tamarisci* (Sw.) Müll. Hall. (Stech et al., 2002) from throughout the range proved to be largely identical, a phenomenon that may be explained by long-range dispersal subsequent to continental drift.

In contrast, sequences of *Pyrrhobryum mnioides* (Hook.) Manuel from Australasia and South America were highly variable (McDaniel & Shaw, 2003) leading to a monophyletic lineage split into two well-supported subclades. The five species of the *Alternantes* clade, however, can be clearly separated by morphological characters and are in each case restricted to one subregion of the Gondwanan range.

The results of an earlier study (Groth & Heinrichs, 2003) and the present investigation point on a center of phylogenetic diversity of Plagiochilaceae in Australasia and Southeast Asia. In this region, the Plagiochilaceae are represented by several genera, whereas in South America (Heinrichs, 2002; Heinrichs et al., 2004b) and in Africa (Jones, 1962) the family is limited to *Plagiochila*. In combination with the fact that the basalmost *Plagiochila* clade of this investigation is of Gondwanan range, our data weakly support a possible origin of the family in Australasia and adjacent regions. However, this hypothesis must be verified by an extension of the taxon sampling as well as of the molecular markers. Work to obtain these data is underway.

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MOSSES SURVIVING ON THE EDGE: ORIGINS, GENETIC DIVERSITY, AND MUTATION IN ANTARCTICA[1]

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Abstract

Mosses are the dominant plant species in continental Antarctica, where they sparsely colonize the limited stony coastal areas not permanently covered with snow and ice. The few species of mosses present in Antarctica live "on the edge" for plant life, surviving in some of the harshest conditions on Earth with extremes of cold, drought, light, and wind. Reproduction is usually by local dispersal of vegetative propagules, and colonization of new locations is very difficult; immigration from other land masses appears infrequent. Moreover, in this region mosses are already exposed to increased levels of UV-B irradiation due to the yearly expansion of the ozone hole and global warming. We have been using techniques of molecular genetics (RAPDs and DNA sequencing) to investigate the biodiversity of Antarctic mosses, population relationships, dispersal and colonization, and mutation. We have also used genetics to determine the identity of some mosses that have proven difficult or impossible to characterize due to their phenotypic plasticity in Antarctica. In addition, we have been able to demonstrate dispersal of different species by either wind or water, and to show dispersal over very short distances of a few meters in frost channels. We have initiated investigation of mutagenesis in Antarctic mosses, both by studying relationships of shoots within individual colonies and by analyzing long shoots cut into small sections. For long shoots, where estimates of growth rates are 0.1–1 mm per year maximum, 10 cm shoots probably represent 100 years of growth. We have detected occasional mutations within such shoots, as well as in different shoots of the same colony—the further shoots are apart within the colony, the more mutations we can detect. The combination of RAPDs and DNA sequencing is proving a powerful method for investigation of the mutation, evolution, and colonization of mosses in Antarctica, and we present here the novel results of our recent investigations.

Key words: Antarctica, bryophyte, genetic variation, Heard Island, Macquarie Island, moss, mutation.

Few plants can survive the rigorous environment of continental Antarctica. A very limited range of moss species are the dominant plants, along with some lichens and algae (Smith, 1984, 1993; Walton, 1990). The origins, colonization history, and dispersal of moss colonies in Antarctica and the subantarctic islands are of interest to bryologists, ecologists, and geneticists alike. These plants that grow at the limits of any plant's tolerance of extremes—drought, cold, and wind, as well as light—have some remarkable characteristics that make them useful for genetic studies of dispersal and mutagenesis in living plants (Skotnicki et al., 2000).

At around 78°S, in southern Victoria Land, there are some small isolated coastal regions and islands where barren gravelly ground is exposed for most of the year and where there is occasional meltwater from glaciers and snow or ice. Here a few species of mosses can survive, despite the extremely harsh climate, with several months of continuous light in the summer months (Seppelt & Green, 1998). These colonies survive freeze-dried during the four months of complete darkness in the winter. These Antarctic mosses cannot reproduce sexually due to the harsh environment and short summer growing period, and so colonies originate either from immigrant propagules from other lands (Marshall, 1996; Marshall & Convey, 1997) or from vegetative propagules dispersed locally. For most genera examined, sporophytes have not been found, and when present on a species, sporophytes have been few and immature (Seppelt et al., 1992).

These mosses are also already subjected to increased exposure to UV-B irradiation, with the an-

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nual expansion of the ozone hole (Farman et al., 1985; Kennedy, 1995). With their haploid genomes, lack of sexual reproduction, perennial growth, and extreme isolation from other colonies elsewhere around the world, these mosses appear to provide an ideal model system for investigations of genetic mutation and evolution in vivo. Therefore, we have used techniques of molecular genetics to investigate the genetic diversity of these plants, their origins and dispersal mechanisms, and their potential to respond genetically to climate change (Skotnicki et al., 2000). We have also used molecular genetics to resolve some taxonomic uncertainties (Skotnicki et al., 2001, 2002), since the extreme environment can lead to phenotypic plasticity where morphological appearance can vary simply due to survival in the extremely harsh conditions rather than being due to genetic changes (Seppelt & Selkirk, 1984; Smith, 1999). This phenomenon in Antarctic moss colonies can make morphological identification difficult or even impossible. In some Antarctic mosses, such as *Ceratodon purpureus,* it has even been suggested that the species is so variable that extreme phenotypes could be taken for distinct species (Ochyra, 1998).

Previously we have mainly used the RAPD (Random Amplified Polymorphic DNA) technique to study dispersal and colonization of Antarctic mosses, as well as some within-colony mutation (Selkirk et al. 1998; Skotnicki et al., 1998a–c, 1999, 2000). The RAPD technique has proven to be very useful for these mosses, due to the lack of other genetic information available, the need to sample minute amounts of moss to avoid damage to the unique environment, and the need to compare many different samples quickly and economically to gain an initial overall comparison within and between the different populations (Skotnicki et al., 2000).

DNA sequencing of highly conserved genes, such as the nuclear ribosomal DNA internal transcribed spacer region (ITS1-5.8S-ITS2), has also become a widelyused technique for phylogenetic analyses in a range of plant species (Baldwin, 1992). DNA sequencing of this region has recently been applied to phylogenetic investigations of temperate bryophytes (Bopp & Capesius, 1996; Patterson et al., 1998; Chiang & Schaal, 1999; Longton & Hedderson, 2000; Shaw 2000a; Vanderpoorten et al., 2001; Boisselier-Dubayle et al., 2002), and we have previously applied the technique to a very isolated population of the moss *Pohlia nutans* (Hedw.) Lindb. in Antarctica (Skotnicki et al., 2002). Recently, several other genes including the three *rps*4, *rbc*L, and *trn*F-*trn*L plastid gene regions have been analyzed in mosses and liverworts (Lewis et al., 1997; Buck et al., 2000; Cox et al., 2000; De Luna et al., 2000; Newton et al., 2000; Shaw, 2000b; Cox & Hedderson, 2003; Magombo, 2003; Pederson et al., 2003; Virtanen, 2003), as they are more highly conserved than the nuclear ITS region and more useful for intergeneric comparisons. For some of the Antarctic mosses, we have also sequenced these three plastid regions in addition to the nuclear ITS region to compare the Antarctic genera with those from elsewhere. We report here our novel results of ITS sequence analyses in Antarctic mosses, in combination with RAPD analyses, as the plastid genes sequenced have so far simply confirmed the ITS results obtained.

Materials and Methods

Collection of Samples and DNA Extraction

Moss samples of seven species were analyzed from Antarctica and elsewhere: *Bryum argenteum* Hedw., *Bryum pseudotriquetrum* (Hedw.) Gaertn., Meyer & Scherb, *Ceratodon purpureus* (Hedw.) Brid., *Hennediella heimii* (Hedw.) R. H. Zander, *Sarconeurum glaciale* (Müll. Hal.) Cardor & Bryhn, *Campylopus pyriformis* (Schultz) Brid., and *Pohlia nutans.* Collection details are summarized in Table 1. Colonies of unidentified mosses with morphologically indistinct phenotypes were also analyzed from Antarctica.

Colonies of mosses were sampled at various locations in Victoria Land, Antarctica, including the Dry Valleys, Granite Harbour, Edmonson Point, Mt. Melbourne, and Mt. Rittmann. Samples were also collected at several locations near Casey Station (Bailey and Clark Peninsulas) in Wilkes Land and near Davis Station (Vestfold Hills) in Princess Elizabeth Land, Antarctica. Mosses were also sampled on subantarctic Macquarie Island and Heard Island and in temperate eastern Australia from locations near Sydney, Canberra, and Hobart and in temperate Christchurch, New Zealand. Small moss samples were collected into paper envelopes, air-dried, and stored frozen or at room temperature until use. Some voucher specimens are held at the University of Waikato in Hamilton, New Zealand (WAIK); moss specimens currently in use are held at the Australian National University in Canberra, Australia. Antarctic moss samples must, of necessity, be extremely small to avoid damaging these slow-growing plants in their unique environment. We are just now obtaining, after 20 years, the first proper growth rate data which show that these mosses grow at a rate of 0.1–1 mm maximum per year. Permit conditions for collection are also of necessity very strict, to minimize damage. Thus, many of our moss

Table 1. Moss samples were all collected in Antarctica, unless otherwise described. Unknown mosses are identified as such. The Mt. Erebus sample was only collected as protonema. The GenBank accession numbers are included for the nuclear ITS region sequences.

Taxon	Collection site	Collection No.	No. in Figs.	GenBank accession No. ITS
Bryum argenteum	Beaufort Island, Ross Sea	15f	1	AY611431
	Canada Glacier, Taylor Valley, S. Victoria Land	P171	2	AY611432
Bryum pseudotriquetrum	Canada Glacier, Taylor Valley, S. Victoria Land	P159	1	AY611427
	Granite Harbour, S. Victoria Land	CCC5	2	AY611426
	Crater Cirque, N. Victoria Land (unknown)	RB1		AY611428
Campylopus pyriformis	Mt. Erebus, Ross Island (protonema only)	ME1	1	AY613328
	Mt. Melbourne, N. Victoria Land	T845	2	AY613329
Ceratodon purpureus	Granite Harbour, S. Victoria Land	CC76	1	AY156590
	Edmonson Point, N. Victoria Land	Epa	2	AY156591
	Crater Cirque, N. Victoria Land (unknown)	RB2		AY613336
	Casey Station, Wilkes Land (unknown)	SR2		AY156592
	Canberra, Australia	Can1		AY156586
	Mt. Banks, Sydney, Australia	MB4		AY156587
	Atlas Cove, Heard Island	ADT #10961		AY156588
	Bauer Bay, Macquarie Island	S41		AY156589
Hennediella heimii	Beaufort Island, Ross Sea	ADT #20336	1	AY613330
	Cape Chocolate, S. Victoria Land	CC16	2	AY613331
Pohlia nutans	Mt. Rittmann, N. Victoria Land	K2	1	AF479318
	Mt. Melbourne, N. Victoria Land	RB3	2	AY484390
Sarconeurum glaciale	Scott Base, Ross Island, Ross Sea	P522	1	AY613332
	Canada Glacier, S. Victoria Land	P185	2	AY616851
	Scott Base, Ross Island, Ross Sea	P510		AY616852
	Kay Island, N. Victoria Land	A563, AAS #09973		AY616853
	Edmonson Point, N. Victoria Land	A573,		AY613333
	Prince Charles Mountains, Mac.Robertson Land	A556		AY616854
	Mossel Lake, Princess Elizabeth Land	A557		AY616855
	Vestfold Hills, Princess Elizabeth Land	V2		AY616856
	Vestfold Hills, Princess Elizabeth Land	V6		AY616857
	Henriksenskjera, Dronning Maud Land	A568, AAS #10066A		AY616858
	Puerto Natales, Patagonia, Chile	SG5		AY616859
Sarconeurum tortelloides	Alexander Island, George VI Sound	AAS #00042		AY616860

samples consist of only a few shoots, and herbarium voucher specimens cannot be lodged while we are still analyzing them.

Herbarium specimens were obtained from the British Antarctic Survey herbarium (AAS) as dried samples and were treated in the same way as other specimens for DNA extraction.

DNA was extracted from the top 3 mm of single moss shoots (Skotnicki et al., 1998a). Briefly, the single shoots were ground in extraction buffer using an electric drill fitted with a sterilized glass bit, extracted with chloroform/isoamyl alcohol, RNase-treated, and precipitated with isopropanol. After rinsing the DNA pellets in 70% ethanol, they were resuspended in 15 µl sterile water and stored at 4°C until used for PCR or RAPD amplification.

RAPD AMPLIFICATION AND ANALYSIS

Moss DNA was amplified by PCR in a microtiter plate (Rafalski & Tingey, 1993; Skotnicki et al., 1998c; Williams et al., 1993). RAPD primers OP-B8, OP-C4, OP-C5, OP-C13, and OP-C14 (Operon Technologies, Inc., Alameda, California, U.S.A.) were used for amplification of *Ceratodon purpureus* DNA (Skotnicki et al., 1998b, 2000).

PCR reactions were incubated as previously described (Skotnicki et al., 1998c), electophoresed through 1.5% agarose gels, stained in ethidium bromide, and photographed under UV light (Sambrook et al. 1989).

A total of up to 153 RAPD bands was scored as present or absent on gels, with PCR reactions being done in duplicate or triplicate to confirm reproducibility of banding patterns. Control reactions were also included to ensure no contamination by fungal or other DNA was a cause of observed differences in banding patterns. The RAPDistance computer program (Armstrong et al., 1995) was used to compare the patterns of DNA fragments pairwise, to produce a distance matrix. From the distance matrix a neighbor-joining tree was calculated (Saitou & Nei, 1987; Jin, 1988; Studier & Keppler, 1988; Ferguson, 1990; Armstrong et al., 1995).

RAPD ANALYSIS OF MUTATION

For analysis of mutation within single colonies, a single colony of *Ceratodon purpureus* (CC76, specimen held at the Australian National University) was selected at Granite Harbour in southern Victoria Land. The colony was some 10 cm in diameter, with approximately 600 shoot tips per square centimeter on the surface. Single shoots were sampled in a grid pattern across the surface, taking 18 shoots adjacent to each other and 18 shoots each at 2 mm, 4 mm, 7 mm, and 10 mm apart. The top 3 mm of each shoot was used for DNA extraction and analysis. RAPD primers were used for DNA amplification, and a total of 153 distinct scorable DNA bands was obtained for the 90 shoots, enabling comparative analysis.

For initial analysis of possible mutations along the length of single shoots, another single colony of *Ceratodon purpureus* was sampled at Granite Harbour. The colony chosen was well isolated from other mosses, protected in a corner between two boulders, and supplied with occasional meltwater runoff from a glacier above it. Thus, it was located in a relatively luxurious spot, and appeared to be an extremely old colony some 30 cm in diameter, with shoots up to 10 cm in length. Growth of moss colonies in Antarctica is estimated to occur at a rate of 0.1–1 mm per year, maximum, so that this colony had shoots potentially 100 years old or more (Selkirk & Skotnicki, unpublished data).

Individual shoots were very fragile, but by soaking a small sample of the colony in sterile deionized water it became possible to gently tease out single shoots up to 75 mm long. Five such shoots were carefully cut into 1 mm sections, and DNA was extracted from the individual sections for RAPD analysis with four primers. A total of 63 RAPD bands was obtained on gels, and these were scored and analyzed for each shoot segment.

DNA SEQUENCING AND PHYLOGENETIC ANALYSIS OF rDNA ITS REGION

DNA was isolated as described above from several different species of moss, including *Ceratodon purpureus, Bryum argenteum, B. pseudotriquetrum, Hennediella heimii, Pohlia nutans, Sarconeurum glaciale,* and *Campylopus pyriformis.* The ITS1-5.8S-ITS2 nuclear ribosomal DNA region (Baldwin, 1992) was amplified using the primers ITS1 and ITS4 (White et al., 1990). Amplification and subsequent sequencing conditions were as described by Skotnicki et al. (2002). The amplified products were sequenced using primers ITS1, ITS4, and the internal primer ITS2 (White et al., 1990). Chromas software (Technelysium Pty Ltd.) was used to edit sequencing chromatograms and assemble the complete sequence of the ITS1-5.8S-ITS2 region and partial sequences of the flanking 18S and 26S ribosomal DNA genes.

The ITS region was amplified and sequenced from seven samples of *Ceratodon purpureus* collected from Granite Harbour, Edmonson Point, and Casey (continental Antarctica), from subantarctic Macquarie and Heard Islands, and from Sydney

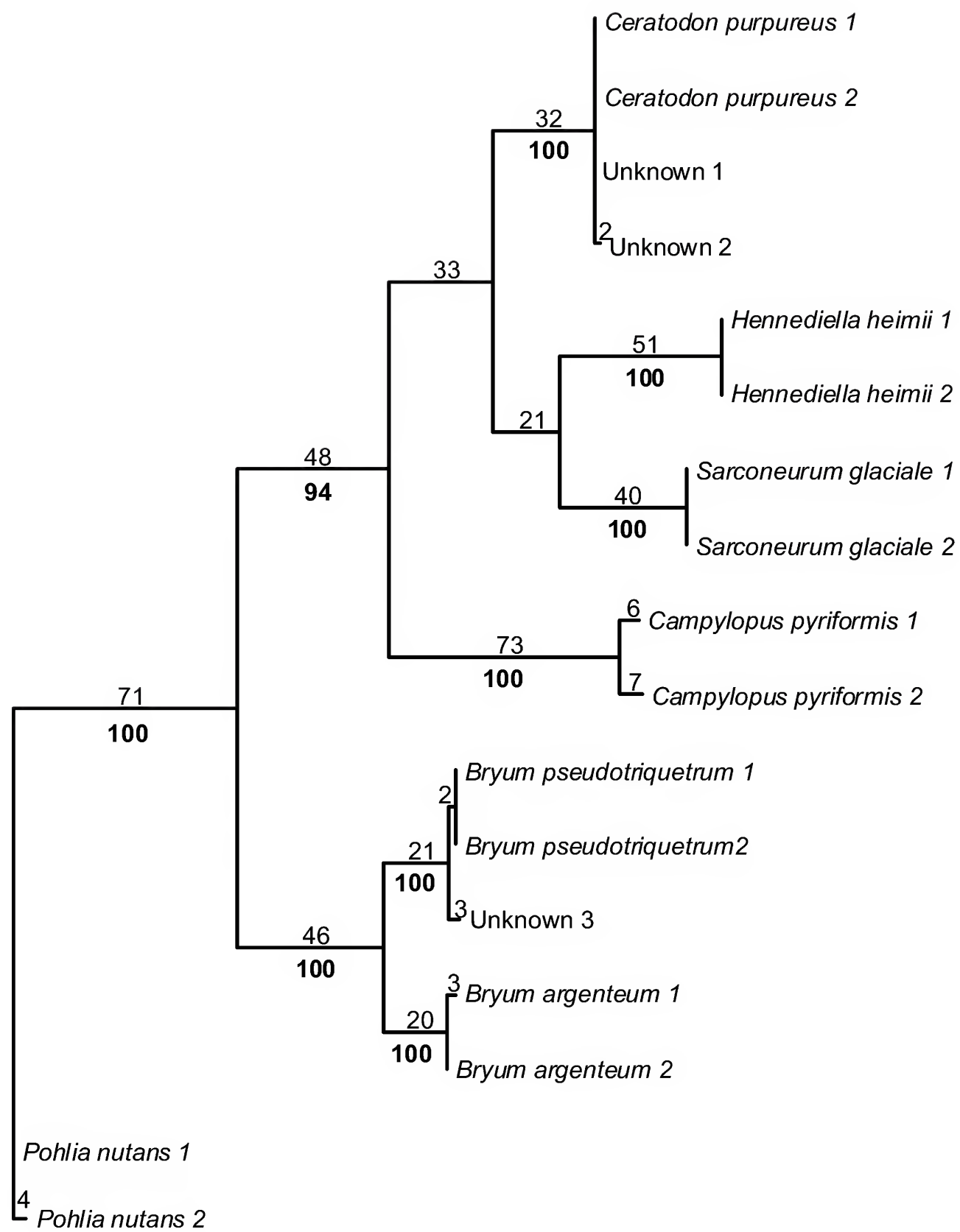

Figure 1. Dendrogram produced from a PILEUP alignment of the conserved nuclear ribosomal RNA ITS1-5.8S-ITS2 sequences for seven different species of moss found in the Ross Sea region of Antarctica (two examples of each species, plus three unidentified samples): *Campylopus pyriformis, Ceratodon purpureus, Pohlia nutans, Hennediella heimii, Sarconeurum glaciale, Bryum argenteum,* and *Bryum pseudotriquetrum.* Note the separation of different species and characterization of three samples of previously unknown identity: two samples of *Ceratodon purpureus,* one from Casey and another from Crater Cirque, and one specimen of *Bryum pseudotriquetrum* from the vicinity of Casey.

and Canberra (Australia). For the Casey sample, only a partial ITS2 sequence was obtained. Sequence data were deposited with GenBank, and accession numbers have been allocated (Table 1). Comparison of the *C. purpureus* sequences was done using the PILEUP and ClustalW programs in ANGIS (Australian National Genome Information Service; Thompson et al., 1994). To select suitable moss species as outgroups for rooting the phylogenetic tree, *C. purpureus* ITS sequences were compared with other known moss sequences for the same DNA region using the BLASTN program via ANGIS and NCBI (National Center for Biotechnology Information; Altschul et al. 1997). *Pottia truncata* (Hedw.) Bruch & Schimp. and *Takakia lepidozioides* S. Hatt. & Inoue were selected as outgroups on the basis of having sequences most similar to the *C. purpureus* sequence. Their sequences and that of a European isolate of *C. purpureus* were obtained from GenBank at NCBI.

ITS sequences were aligned from the seven *C. purpureus* isolates alone and also from the *C. purpureus* isolates with the two outgroup species. Sequences were first aligned in BioEdit version 4.7.8. using the E ClustalW program and adjusted by eye thereafter, and gaps were inserted to maintain areas of nucleotide homology. The aligned files were exported through MacClade (Maddison & Maddison, 1992) for analysis in PAUP* 4.0b4a (Swofford, 1998). The ITS alignment, which included the two outgroups, showed a high level of divergence among the *T. lepidozioides*, *P. truncata*, and the *C. purpureus* group, and there are a number of areas of ambiguity. Since these two outgroups were used solely for rooting the phylogenetic tree, it was not considered necessary to remove the ambiguous regions before further analysis.

Most parsimonious trees were determined using a heuristic search with 100 replicates of random taxon entry, tree-bisection-reconnection branch swapping, and the MulTrees option (keeping multiple, most parsimonious trees). Bootstrap analyses (1000 replicates) were done to determine clade support (Felsenstein, 1985).

Similar sequencing of the ITS region of other Antarctic moss species, and alignment using similar methods, enabled comparison of the different species and identification of some "unknown" mosses that could not be identified by morphology alone. This included analysis of two samples of each of the seven dominant moss species found in Victoria Land to assess whether molecular methods could be used to reliably distinguish the seven species in the absence of characteristic morphological features.

In addition, DNA sequencing of the ITS region was used to analyze in more detail the relationships and possible dispersal of populations of *Sarconeurum glaciale* and of two other moss species found only as extremely small and isolated populations on geothermally warmed ground near the summits of Victoria Land volcanoes.

Results

ITS sequencing for taxonomic identification of Antarctic mosses

We have sequenced the nrDNA ITS1-5.8S-ITS2 region (including the 3′ terminal nucleotides of the 18S gene, the complete ITS1 region and 5.8S gene, and almost all of the ITS2 region) from the seven dominant moss species found in the Ross Sea/Victoria Land region of continental Antarctica. This region was sequenced for at least three, and often six or seven samples from each of the seven moss species. Usually, 850–1000 nucleotides were analyzed, depending on the length of the ITS region; sequencing with primers ITS2, ITS4, and ITS5 enabled the full sequence to be constructed.

In Figure 1, the complete sequences (except for the last few nucleotides of ITS2) have been used to construct a dendrogram of relatedness for these species. Figure 1 shows that there is genetic variation between different isolates of the same moss species. Although only two samples of each species are included in this dendrogram for clarity, similar levels of variation were observed in many other samples. Overall, samples from sites very close together were much more likely to have identical sequences, and the further apart samples were collected, the more likely they were to exhibit more differences within their nucleotide sequences.

Figure 1 also includes three mosses that had not previously been identified by morphological means. These three unknown samples were readily identified by comparison of their ITS1-5.8S-ITS2 sequences with those of the seven species from Antarctica documented here. One specimen from Casey station and one from Crater Cirque (Victoria Land) were both characterized as *Ceratodon purpureus*, and another from near Casey was identified as *Bryum pseudotriquetrum*.

However, comparison of a much smaller region of the sequence was sufficient for identification of these species and for distinction of the seven dominant species of the Ross Sea region. When the three unknown samples were included with known species in an alignment of only 100 nucleotides from the ITS2 region, these mosses were readily identified as *Ceratodon purpureus* and *Bryum pseudotriquetrum* (Figure 2). This result demonstrates that this small, readily amplified and conserved section of the genome is sufficient for identification and characterization of the seven Antarctic moss species analyzed.

ITS sequence diversity in Antarctic moss species—*Campylopus* and *Pohlia* from geothermally warmed ground

The ITS1-5.8S-ITS2 sequences of two specimens of *Campylopus pyriformis* have been determined and were identical despite being from different colonies in distinct sites some 200 m apart and separated by snow and ice on Mt. Melbourne (Broady et al., 1987). The ITS sequence of the only other known Antarctic *C. pyriformis* specimen has also been determined; this sample was collected over 20 years ago from the summit of another volcano, Mt. Erebus, some 400 km south of Mt. Melbourne on

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Bryum argenteum 1  ATATTCTAAG TCCC-CAAGA CTGAGGACCC TTA-TTTTGA CCCGAGAGTT CGAG------ TCCTCCGG-- -GG-CTCGAG CACGAGTTGG ATCTAAAACT
Bryum argenteum 2  ATATTCTAAG TCCC-CAAGA CTGAGGACCC TTA-TTTTGA CCCGAGAGTC CGAG------ TCCTCCGG-- -GG-CTCGAG CACGAGTTGG ATCTAAAACT
B. pseudotriq 1    TTTTTCTAAG TCCCACAAGA CTTGAGACCC TTAATTTTGA CCCGGGAGTT TGGGCTCACT TCCTCGGGAG TGGGCTCGGA CACGAGTTGA ATCTAAT-CT
B. pseudotriq 2    TTTTTCTAAG TCCCACAAGA CTTGAGACCC TTAATTTTGA CCCGGGAGTT TGGGCTCACT TCCTCGGGAG TGGGCTCGGA CACGAGTTGA ATCTAAT-CT
Unknown 1          TTTTTCTAAG TCCCACAAGA CTTGAGACCC TTAATTTTGG CCCGGGAGTT TGGGCTCACT TCCTCGGGAG TGGGCTCGGA CACGAGTTGA ATCTGAA-TC
Campylopus 1       ----CTTA-G AACC--GGGC GCGATCATCA TCCTCCTCCT TAAGCAT--- CATC------ ------ATCA TCA-----TG ----AGTTGT GA--AAAATA
Campylopus 2       ----CTTA-G AACC--GGGC GCGATCATCA TCCTTCTCCT CAAGCAAG-- CATC------ ------ATCG CGA-----GT ----TGTTGT GA--AAAATA
Ceratodon 1        ----CGATTG CGCA--ATGC ATCAACACTT TCAATATGAC TGAGTATAAA AATT------ ------TCGG CGCCTTCGGC ----GCCCGA GTT-GTAATA
Ceratodon 2        ----CGATTG CGCA--ATGC ATCAACACTT TCAATATGAC TGAGTATAAA AATT------ ------TCGG CGCCTTCGGC ----GCCCGA GTT-GTAATA
Unknown 2          ----CGATTG CGCA--ATGC ATCAACACTT TCAATATGAC TGAGTATAAA AATT------ ------TCGG CGCCTTCGGC ----GCCCGA GTT-GTAATA
Unknown 3          ----CGATTG CGCA--ATGC ATCAACACTT TCAATATGAC TGAGTATAAA AATT------ ------TCGG CGCCTTCGGC ----GCCCGA GTT-GTAATA
Hennediella 1      ----CAAG-- ---------- AGGA-AACTC TCCCTCTCG- AAAGTTAG-- ---------- ------GGAG AGC-----GT ----ACTCGA GTT-GTAATA
Hennediella 2      ----CGAG-- ---------- AGGA-AACTC TCCCTCTCG- AAAGTTAG-- ---------- ------GGAG AGC-----GT ----ACTCGA GTT-GTAATA
Pohlia 1           ----TATATG GCCC--GGGA GTATGAGTGG TGCCGAGTGC GGGGCGGGCT CGTCC----- CGCTCCGTCA CGAGGCGCGC TCAAAACCGA GTT-GTGATT
Pohlia 2           ----TATATG GCCC--GGGA GTATGAGTGG TGCCGAGTGC GGGGCGGGCT CGTCC----- CGCTCCGTCA CGAGGCGCGC TCAAAACCGA GTT-GTGATT
Sarconeurum 1      ----CAAACT CTCT--ACCT ACCT-CACTT ACCTTTTGG- TGAGTGAGCA AGT------- ------AAAG TGA-----GT ----GAGTGA GTT-GTAATA
Sarconeurum 2      ----CAAACT CTCT--ACCT ACCT-CACTT ACCTTTTGG- TGAGTGAGCA AGT------- ------AAAG TGA-----GT ----GAGTGA GTT-GTAATA
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Figure 2. Comparison of 100 bp of the conserved nuclear ribosomal RNA ITS1-5.8S-ITS2 nucleotide sequences of seven different Antarctic moss species and three unknown moss specimens as in Figure 1, showing that this small region is sufficient for identification of unknown mosses as *Ceratodon purpureus* and *Bryum pseudotriquetrum* (abbreviated *B. pseudotriq*). The alignment was produced by ClustalW.

Ross Island (Broady, 1984). As it was originally collected as protonemal growth that could not be differentiated in culture, RAPD analysis was the first means of identification for this specimen, enabling it to be identified as *C. pyriformis* (Skotnicki et al., 2001). Although it was reasonably similar to the Mt. Melbourne population by RAPD comparison, the ITS sequence had several distinct differences from the Mt. Melbourne sequences, as indicated in the dendrogram in Figure 1. However, identification of the Mt. Erebus moss has been further confirmed by DNA sequencing of the ITS region, showing that it is indeed *C. pyriformis,* with 92% of the 840 nucleotides analyzed being identical to the Mt. Melbourne *C. pyriformis* sequence.

Like *Campylopus pyriformis,* the moss *Pohlia nutans* has only been found in Antarctica as a very small population near the summit of a volcano, where it colonizes a few small areas of geothermally warmed ground on Mt. Rittmann (Bargagli et al., 1996). We have previously used RAPDs to demonstrate the close relationships of colonies within this population, and have also used ITS sequencing to show that this population is related to *P. nutans* from the Peninsula region of Antarctica (Skotnicki et al., 2002).

We have now sequenced the ITS region of another moss sample collected from a distinct volcanic site in the region (Bargagli, pers. comm.), and shown that this is also *Pohlia nutans,* very closely related to the Mt. Rittmann population, with just a few different nucleotides in the ITS region. The results are included in the dendrogram shown in Figure 1.

ITS SEQUENCE DIVERSITY IN ANTARCTIC MOSS SPECIES—*SARCONEURUM*

Sarconeurum glaciale occurs in a number of isolated locations around coastal Antarctica where its reproduction is solely vegetative, by means of deciduous leaf tips. This species has only been found outside Antarctica in Patagonia, Chile (Matteri, 1982), where it grows among rocks and on the bark of *Nothofagus antarctica* (G. Forst.) Oerst. trees and reproduces sexually (Greene, 1975; Matteri, 1982). We have previously compared the genetic diversity and population relationships of this moss by RAPD analysis (Skotnicki et al., 1999). The results showed the populations from the four locations to be distinct. Those from Ross Island and eastern Taylor Valley (100 km apart) were closely related to each other, and were more distantly related to the population from Patagonia (over 5000 km away in a straight line, 12,000 km around the coast following the direction of the prevailing westerly wind). These three populations together formed a clade on a dendrogram, distinct from the clade with the more variable Vestfold Hills specimens (2700 km away in a direct line, but 5000 km along the partially ice-free coast; Skotnicki et al., 1999).

To further resolve the relationship of the Vestfold Hills population with those from elsewhere, we have sequenced the nrDNA ITS1-5.8S-ITS2 region from a number of specimens of *S. glaciale* to compare the genetic diversity in more detail. Results for typical isolates are shown in Figure 3. It is clear from the dendrogram that the Vestfold Hills population is quite different from the other samples; several other specimens from four distinct locations in the Vestfold Hills all gave practically identical ITS sequences to those isolates shown in Figure 3. When the ITS sequences were aligned in BioEdit, the Vestfold Hills mosses only shared approximately 60% sequence similarity with any of the other *S. glaciale* populations. One unknown moss specimen (sample A556) from the Prince Charles Mountains is included in Figure 3, as it was thought to be possibly a *Sarconeurum glaciale* specimen from morphological examination. However, the sequencing results indicate it to be another species, even

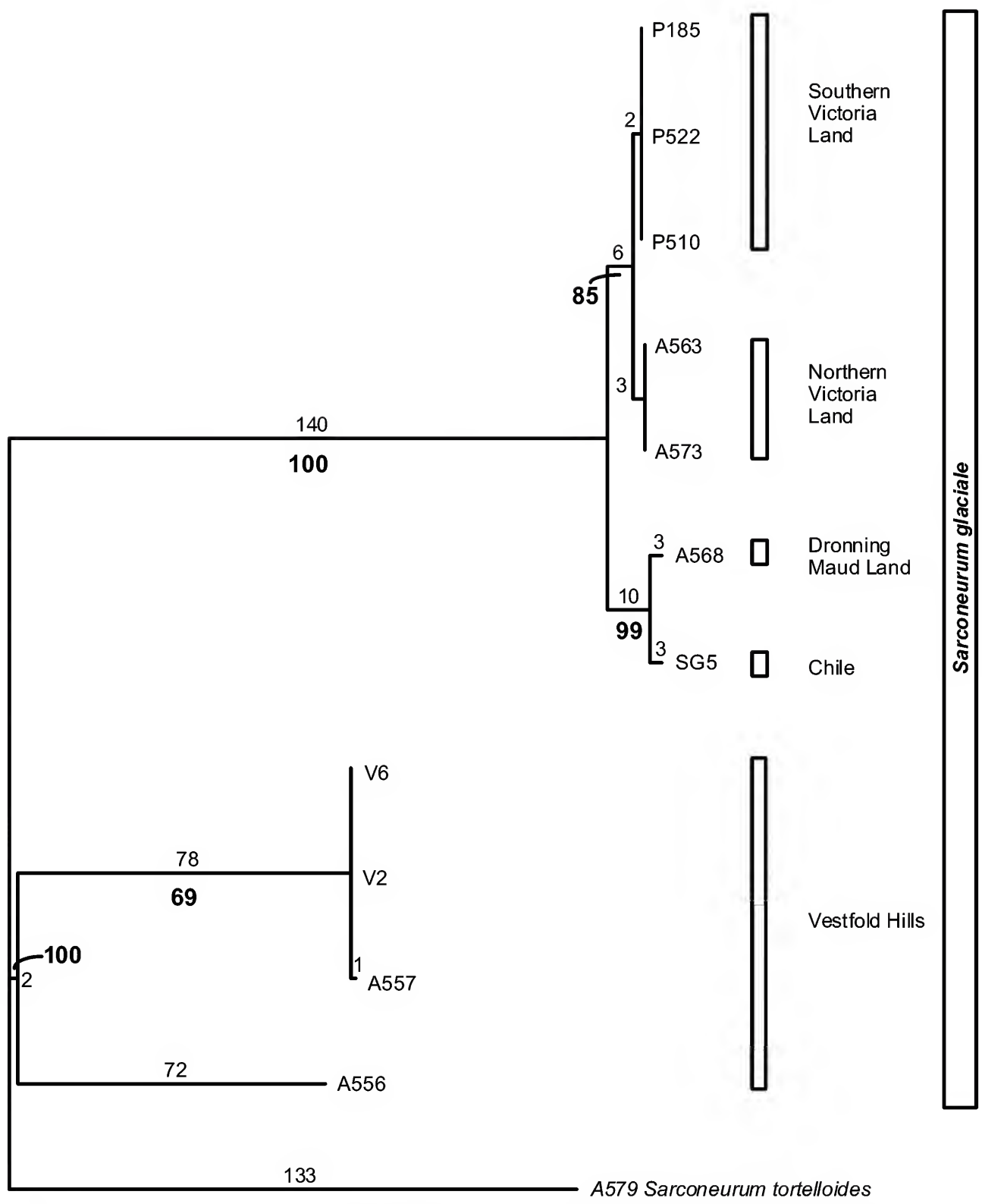

Figure 3. Dendrogram produced from a PILEUP alignment of the conserved nuclear ribosomal RNA ITS1-5.8S-ITS2 sequences for *Sarconeurum glaciale* specimens from Ross Island and the Taylor Valley in southern Victoria Land, Edmonson Point and Crater Cirque in northern Victoria Land, Patagonia in Chile, Dronning Maud Land, and the Vestfold Hills (see Table 1). One unknown moss specimen from the Prince Charles Mountains is included (A556), as it was thought to be possibly a *Sarconeurum glaciale* specimen. *Sarconeurum tortelloides* S. W. Greene from Chile is included as an outgroup.

more distantly related than the Vestfold Hills population.

Currently, we are sequencing three other genes (*rps*4, *rbc*L, and *trn*L) from these samples to determine whether cryptic speciation is occurring in the Vestfold Hills population; the specimens were identified by three bryological taxonomists as being typical *S. glaciale* samples based on morphological characteristics. Preliminary results indicate approximately 96% similarity in the *rbc*L gene for *S. glaciale* from the Vestfold Hills and Victoria Land.

ITS SEQUENCE DIVERSITY IN ANTARCTIC MOSS SPECIES—*CERATODON PURPUREUS*

Sequencing of a 780 bp DNA band amplified from seven isolates of *Ceratodon purpureus* revealed the 3′ end of the 18S rRNA gene, the complete ITS1-5.8S-ITS2 region, and the 5′ end of the 26S rRNA gene. ITS sequences from seven *C. purpureus* samples have been deposited with GenBank, as detailed in Table 1.

Comparison of these *C. purpureus* sequences (us-

ing the ClustalW program) showed more variation in the ITS1 intergenic spacer region (length 260–284 bp) than in the ITS2 intergenic spacer region (length 294–303 bp), with several small substitutions and indels (insertions and deletions). The 5.8S rRNA gene was conserved within the *C. purpureus* isolates analyzed from Australia, continental Antarctica, and subantarctic Macquarie Island. There was a single nucleotide difference in both the Heard Island and European isolates, at different positions within the 5.8S sequence.

The Heard Island isolate had a large deletion of 60 bp compared with all other isolates. Numerous small indels (1–7 bp) were observed in the two Australian isolates compared with the other *C. purpureus* sequences. The Macquarie Island and Antarctic isolates were very similar in the ITS region, with the Granite Harbour isolate having a 4 bp indel. A second specimen from Granite Harbour was also analyzed and gave an identical sequence to the one used to construct the dendrogram shown in Figure 1.

The alignment of *C. purpureus* ITS sequences was used to produce a dendrogram (Fig. 4). One other European *C. purpureus* sequence was available in the NCBI database (*Capesius Y17136*; Capesius, 1998), and this was included for comparison. Phylogenetic analysis of the ITS sequences revealed variation in the different geographic isolates of *C. purpureus*, and two clades were resolved. One clade included all the continental Antarctic and subantarctic isolates with the European specimen. Within this clade, the European sequence appeared most closely related to that of the Heard Island isolate. The other clade contained the two Australian isolates, distinct from the Antarctic and subantarctic isolates. However, all of the *C. purpureus* samples were very similar, when compared with the two species used as outgroups (Fig. 4).

MUTATION WITHIN A SINGLE COLONY OF *CERATODON PURPUREUS*

We have previously reported that genetic diversity can be detected within single colonies of Antarctic mosses, including *Ceratodon purpureus* (Skotnicki et al., 2000). Recently, we have analyzed genetic diversity in more detail, by screening individual shoots sampled across a grid pattern from a single colony. This colony was chosen because the shoots were very densely packed, minimizing the possibility of immigrant shoots with different genetic profiles masking the true mutational events within the colony. No apparent immigrant shoots were observed in the shoots tested from this colony, nor were any shoots of other moss species seen, although we detected immigrant *C. purpureus* shoots within one other *C. purpureus* colony tested; these immigrant shoots had a distinct RAPD banding profile quite different form the occasional changes observed within the 90 shoots from the dense colony (Skotnicki et al., 2000).

Results obtained for 90 shoots from one colony are summarized in Figure 5, demonstrating that RAPD band differences do occur within a single colony. Figure 5 shows that adjacent shoots were almost always identical, whereas about 5% of the shoots 10 mm apart had detectable variation within the 153 bands analyzed. Moreover, a higher number of bands were different in the more distant shoots than in the close ones—shoots 2 mm apart, if different, were most likely to differ by a single RAPD band change, whereas shoots 7–10 mm apart, if different, were more likely to differ by 1 to 5 RAPD bands.

MUTATION ALONG A SINGLE LONG SHOOT OF *CERATODON PURPUREUS*

As mutation appeared to be the cause of genetic differences between shoots within a single colony of *Ceratodon purpureus*, we analyzed variation along single shoots of a single, very isolated but large and apparently ancient colony of this moss growing in a particularly protected situation at Granite Harbour, southern Victoria Land. The majority of colonies in the vicinity had shoots up to 3 cm long, whereas the protected colony had shoots up to 10 cm long in the center of the colony.

Almost all of the banding patterns were identical along the length of each shoot. However, a few differences were observed, and one such band change is shown in Figure 6. Altogether, in the five shoots tested, a total of seven RAPD band changes were observed, indicating mutation had occurred in these shoots during their growth. Where a band change occurred, it usually remained constant in the younger sections up the shoot, although in two instances the changed band reverted to the original size RAPD band a few sections further up the shoot.

Discussion

Antarctica is remote and inhospitable: it is the coldest, driest, highest, and windiest continent, and during the winter months, it is dark and isolated—surrounded by an area of sea ice larger than the continent itself. The few moss species that can colonize and survive the rigors of continental Antarctica grow in probably the harshest environment on

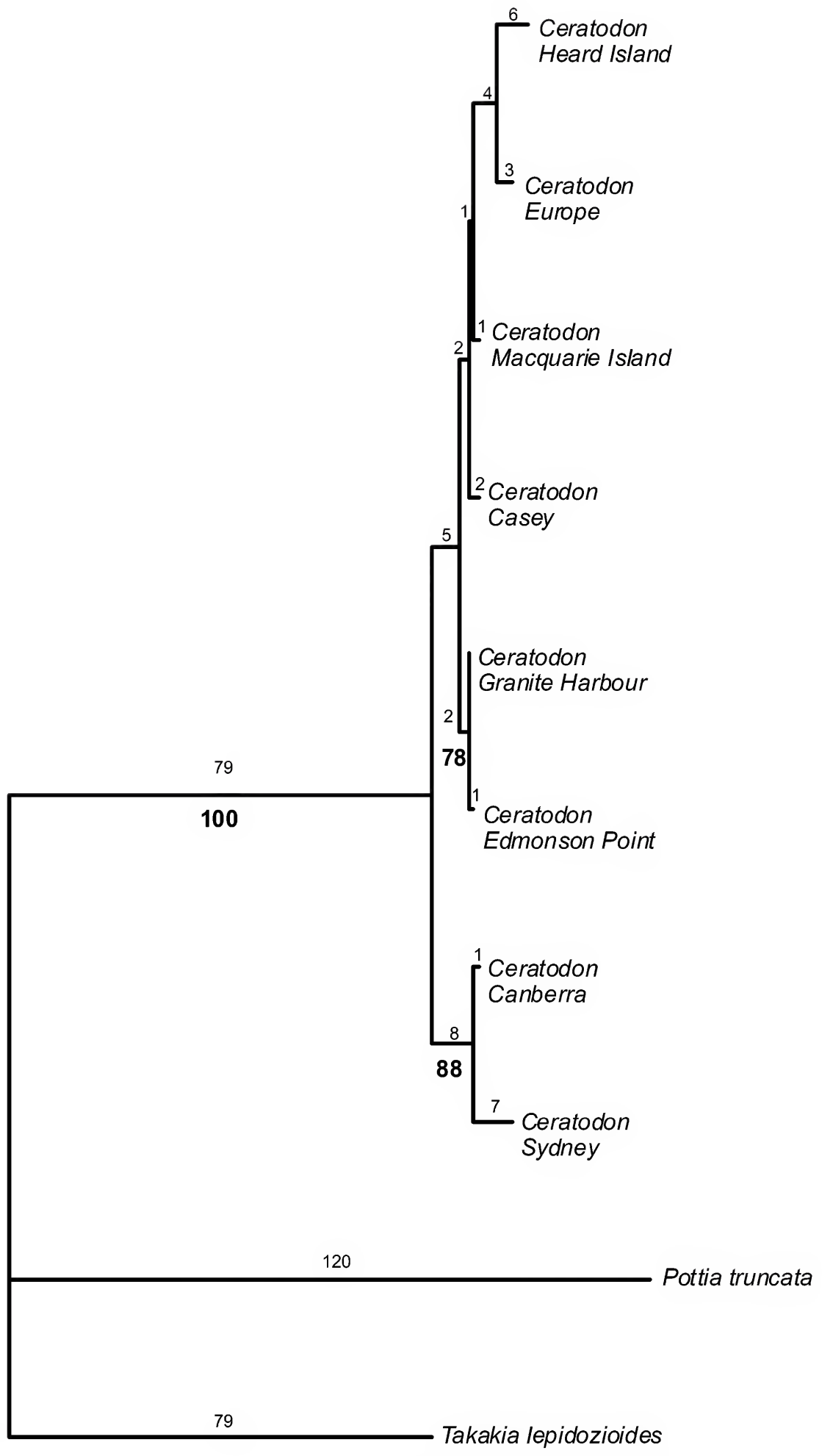

Figure 4. One of two equally most parsimonious trees from the ITS analysis of *Ceratodon purpureus* and 2 outgroup species *Pottia truncata* and *Takakia lepidozioides* (L = 321, CI = 0.953, and RI = 0.808). All samples shown are of *C. purpureus* except for the two outgroup samples; GenBank accession numbers are given in Table 1. The tree is shown with branch lengths equal to the number (above line) of estimated substitutions (ACCTRAN optimization), and numbers in bold below the branches are bootstrap percentages (> 50%). The division of *C. purpureus* isolates into two clades can be seen, one with Australian isolates (Canberra and Sydney) and the other with those from Antarctica, subantarctic islands, and Europe.

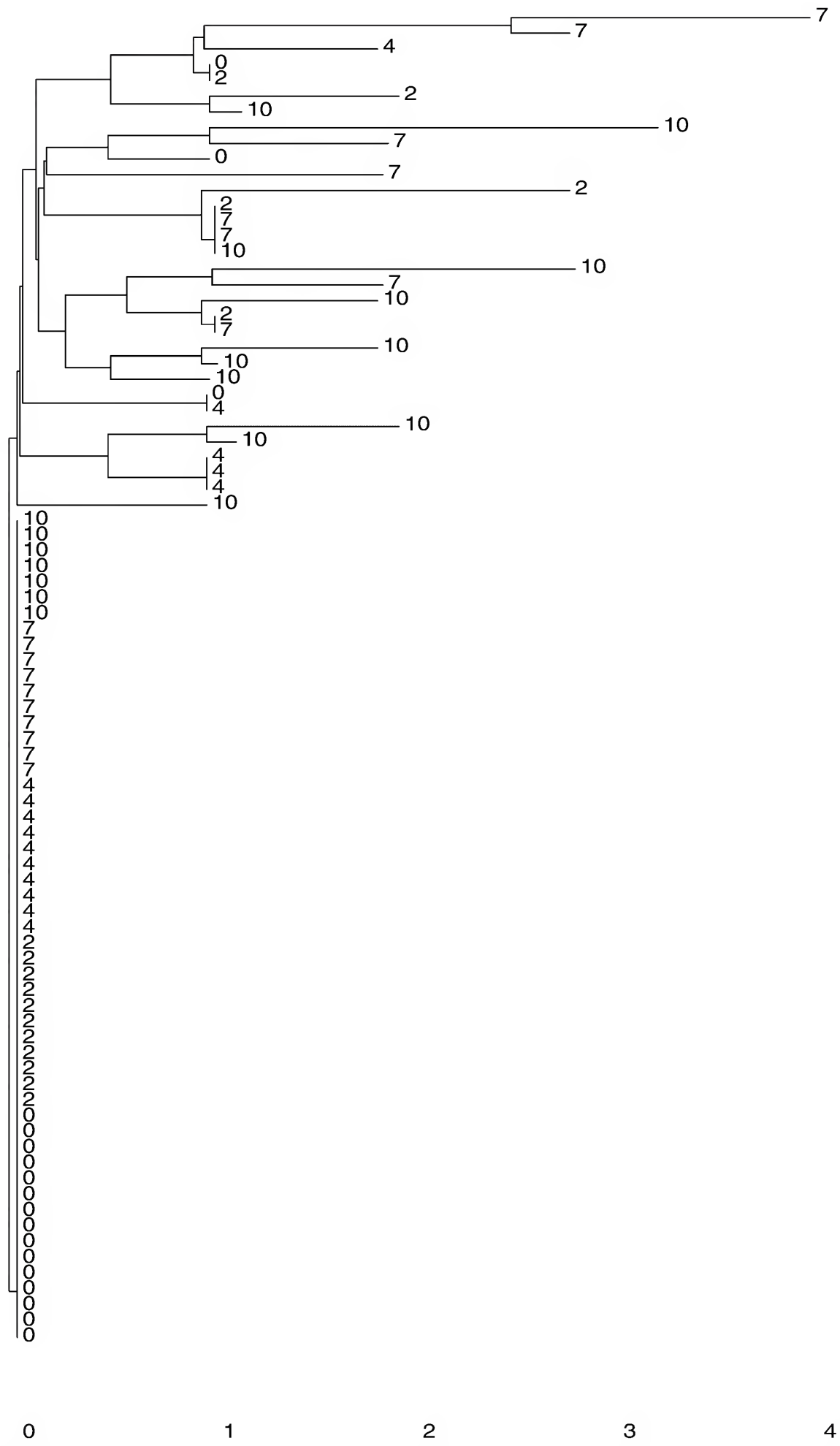

Figure 5. Neighbor-joining tree of relatedness for shoots from a single colony of *C. purpureus* from Granite Harbour. Within this dendrogram, the majority of shoots show no differences, but shoots with one, two, three, four, or five RAPD band differences can be detected according to the clustering of samples; numbers at the bottom of the dendrogram indicate approximate number of genetic alterations within the 153 bands analyzed. Shoots sampled adjacent to each other are labeled 0, those 2 mm apart are labeled 2, 4 mm apart are labeled 4, 7 mm apart are labeled 7, and 10 mm apart are labeled 10.

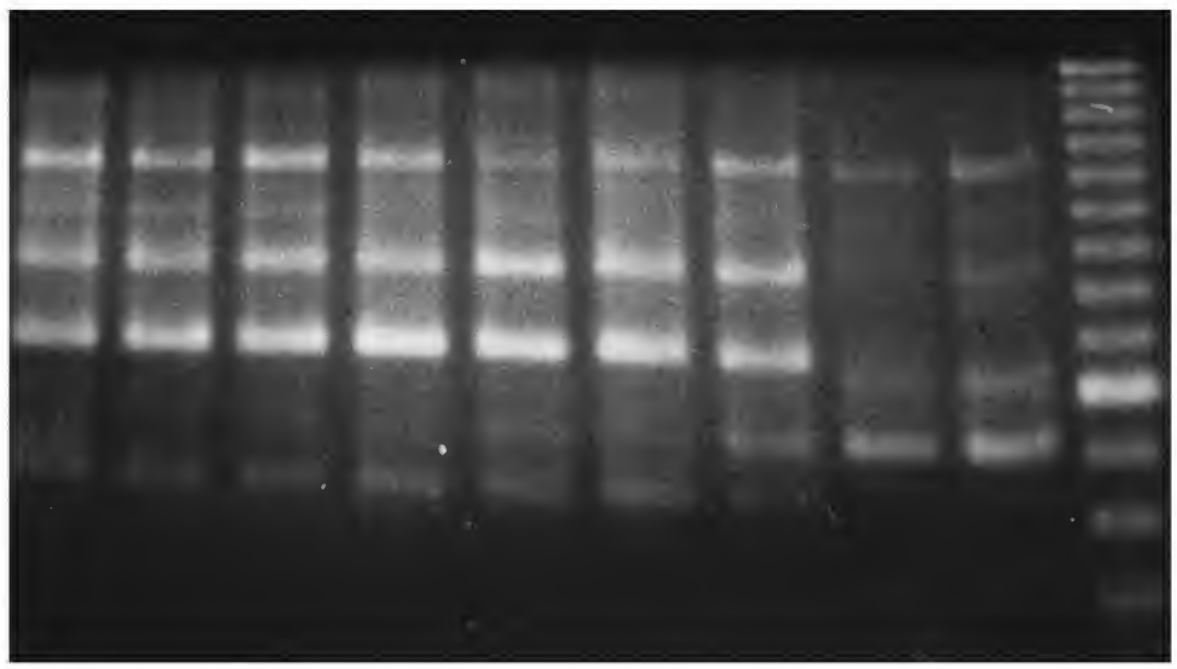

Figure 6. Agarose gel photograph showing RAPD banding patterns for 1 mm segments of a single old stem of *Ceratodon purpureus* from Granite Harbour, Victoria Land. Note the band changes progressing up the stem (left to right). Only the RAPD bands for 9 segments of the stem are shown; a total of 75 segments were analyzed for this shoot, but this was the only part of the length that showed a change in banding pattern, visible in lanes 7, 8, and 9. A total of 63 bands were screened, with 4 primers in separate reactions.

Earth (Walton, 1990), and are also subjected to increased levels of UV irradiation due to the annual expansion of the ozone hole (Farman et al., 1985; Clilverd et al., 2003). Because these mosses are perennial plants with multiple shoots that arise from single apical meristematic cells, have a haploid genome, and reproduce vegetatively in the absence of sexual reproduction, they provide an excellent model system for investigation of plant colonization, dispersal, mutation, and evolution. Such a combination of characteristics is not available in other plants, or even in mosses in most other parts of the world (Wyatt, 1994). Thus, the mosses of Antarctica are worthy of genetic investigation, with the potential to reveal significant insights into mutation and speciation.

We have demonstrated that two methods of molecular genetics, RAPD analysis and DNA sequencing, are complementary and can be used beneficially either singly or in combination to characterize Antarctic mosses that cannot be identified taxonomically by classical morphological means. Similar use of combinations of morphological and genetic characteristics have also been used to investigate temperate bryophytes and ferns (Boisselier-Dubayle & Bischler, 1994; Boisselier-Dubayle et al., 1995; Schneller et al., 1998; Vanderpoorten et al., 2003; Virtanen, 2003). A moss found only as protonemal growth near the summit of volcanic Mt. Erebus has been identified as *Campylopus pyriformis,* and despite differences of 8% in the nucleotide sequence compared with the only other known Antarctic *C. pyriformis* population (Skotnicki et al., 2001), it appears most likely that they are derived from one another. The nearest known alternative source for colonization is New Zealand, several thousand kilometers away, and samples analyzed from two populations there were very different (Skotnicki et al., 2001). It would appear that mutation can occur relatively easily in Antarctic *C. pyriformis,* as shown by the extent of RAPD variation within an extremely remote and small population on Mt. Melbourne. Genetic variation among shoots from single colonies in this population (Skotnicki et al., 2001) also lends strong support to mutagenesis being the cause of genetic differences. It is tempting to speculate that *C. pyriformis* on Mt. Erebus has mutated and lost the genetic capability to differentiate into the morphologically recognizable gametophyte.

ITS sequencing appears to be a highly appropriate method for identification of some of these Antarctic mosses, especially when growth is so slow and damage to colonies must be minimized. A single 2 mm shoot provides sufficient DNA for sequencing of several genes, as well as some RAPD analysis. We have used this method to identify isolates of *Ceratodon purpureus, Bryum pseudotriquetrum, Sarconeurum glaciale, Pohlia nutans,* and *Campylopus pyriformis* (see Figs. 1, 3); currently, we are extending the method to identify a wider range of Antarctic mosses using not only ITS but also *rbc*L, *rps*4, and *trn*L-F genes, which have proved useful for temperate bryophytes (Buck et al., 2000; De Luna et al., 2000; Shaw, 2000b; Cox & Hedderson, 2003; Shaw et al., 2003). Given that moss phenotypes can be very plastic in the Antarctic environment, defying attempts to identify

them morphologically, DNA sequencing is likely to be used more widely for taxonomic purposes. Because there are so few moss genera in continental Antarctica, it appears that even a very short sequence of 100 or so nucleotides may give a very useful indication of the likely species. However, it will be important to bear in mind the possibility of mutation and cryptic speciation, as perhaps suggested by our investigations of *Sarconeurum glaciale*.

We have used RAPDs to analyze methods of dispersal of Antarctic mosses, and have shown that both wind and water are important. Different species appear more dependent on either water (e.g., shoot tips of *Bryum argenteum*; Selkirk et al., 1998) or wind (e.g., *Hennediella heimii*; Selkirk et al., 1998). In addition, we have shown that moss propagules can be blown tens of kilometers over the ice cap, to be deposited at the front of a retreating glacier where meltwater is occasionally present (e.g., *Bryum pseudotriquetrum*; Skotnicki et al., 1998c). RAPDs have also proven invaluable for analysis of very short-distance dispersal of *Sarconeurum glaciale* down frost heave channels. These channels can be a few meters in length and accumulate snowdrifts and, thus, occasionally have meltwater; water appears responsible for dispersal downstream, whereas wind appears to be the cause of new colonizations (Skotnicki et al., 1999).

We have confirmed that RAPD results are mirrored by DNA sequencing results, some of which are presented here. Similarly, for analysis of mutation within many comparable DNA samples extracted from a single shoot or colony, the RAPD technique has given excellent results for numerous genes quickly and economically. Thus, it remains the technique of choice for such investigations, whereas DNA sequencing is more suited to detailed analysis of a smaller number of samples. Together the techniques are complementary, providing different insights into mutation, origins, and dispersal of mosses in Antarctica.

This report marks the first use of the widely utilized DNA sequencing technique for phylogenetic analysis of several species of Antarctic mosses. The results shown here are typical of many we have already obtained for seven Antarctic moss species, confirming that for these plants, as for mosses from temperate regions, this part of the genome can be used for both phylogenetic and taxonomic purposes.

DNA sequencing of the ITS region, as well as other genes, in temperate mosses has clearly shown that these haploid plants can maintain higher levels of genetic variation even in conserved multicopy genes than might have been expected (Shaw, 2000a, 2001; Skotnicki et al., 2000). In some temperate species, it has been proposed that this variation could lead to cryptic speciation. The results presented here indicate that levels of ITS sequence variation are also high in Antarctic moss species. As mosses in continental Antarctica appear to reproduce solely by vegetative means, such genetic variation is presumably caused by somatic mutation alone and could be involved in evolution of new species (cryptic or otherwise, over time; Longton & Hedderson 2000; Shaw 2000a, 2001; McDaniel & Shaw, 2003; Shaw et al. 2003) that can specifically withstand the extremely harsh climate of the region.

Sequence analysis of *Sarconeurum glaciale* colonies gave results consistent with those obtained by the RAPD technique (Skotnicki et al., 1999). Of particular interest is the population in the Vestfold Hills, where it appears the species may have already undergone cryptic speciation. This population is some thousands of kilometers distant from other known *S. glaciale* populations, and may have evolved in isolation over thousands of years. The differences in the ITS region amount to some 41%–46% compared with the Victoria Land and Patagonian populations, although these mosses were both classified as *S. glaciale* on morphological grounds (Greene, 1975; Matteri, 1982; Seppelt & Green, 1998). It will be interesting to analyze the DNA sequences of some other more highly conserved genes in the Vestfold Hills mosses for further comparison with other populations, although preliminary results indicate much lower levels of genetic variation in these genes.

Despite the presence of sexually reproducing plants in the vicinity, the level of genetic variation within the Patagonian population was comparable with that in the population from the Ross Sea region, where reproduction is solely vegetative. Genetic variation within the Vestfold Hills population was greater than that in the Ross Island and eastern Taylor Valley populations, as was previously found with other populations of this species from the Ross Sea region (Selkirk et al. 1997; Skotnicki et al., 1999). This may indicate that multiple colonization events, or extensive mutation, have taken place in the Vestfold Hills, compared with perhaps a single original colonization and more limited mutation in the Ross Sea region. Mutation appears capable of creating as much genetic variability in a vegetative population as is found in a more temperate, sexuallyreproducing population. This fascinating possibility is worthy of further investigation.

The potential *S. glaciale* identified on morphological basis from the Prince Charles Mountains appears to be another genus based on DNA sequenc-

ing results. We are now comparing *Coscinodon, Bryoerythrophyllum,* and *Schistidium* specimens from the coastal areas in that part of the Antarctic continent to resolve the identity of the Prince Charles Mountains isolate. It appears that phenotypic plasticity is again masking the true identity, perhaps because of the extreme conditions in this isolated habitat, but genetic methods should enable identification by comparison with known sequences.

RAPD analysis of *Ceratodon purpureus* specimens from a range of Antarctic, subantarctic, and temperate populations showed that significant levels of genetic variation occurred within populations (Skotnicki et al., 1998b). This has now been confirmed and extended with sequencing of the ITS region, and the results indicate that Antarctic and subantarctic isolates form one clade and Australian isolates another. However, the differences detected were relatively small, and they may be the beginning of cryptic speciation (Longton & Hedderson 2000; Shaw 2000a, 2001; Shaw et al. 2002), although the changes were not sufficient to warrant their separation into a separate species as previously proposed (Burley & Pritchard, 1990). Several other genes (*trn*L-F, *rps*4, *rbc*L) from these specimens are currently being sequenced for more detailed comparison.

We have also demonstrated conclusively that mutation can be the cause of genetic diversity in Antarctic mosses, since genetic changes were detected as RAPD band changes within colonies and even occasionally within single shoots. Thus mutation is occurring in these vegetative Antarctic mosses (Newton & Mishler, 1994), with more distant shoots showing more genetic changes. These results also demonstrate that RAPDs have a significant role to play in analysis of within-colony and within-shoot mutagenesis. Although it is difficult to relate the observed genetic changes precisely to growth rate because growth measurements are notoriously difficult to obtain in the harsh conditions and extremely slow-growing Antarctic mosses, it appears that long shoots (10 cm in the colony analyzed) may be 100 years old or more. Similarly, in a colony with 600 shoots/cm^2 each 1 cm in length, one shoot tip 7 mm from another is certainly separated from it by tens of years of growth, if not more. Therefore, analysis of 36 shoots from that colony, each at least 7–10 mm from the next, is actually investigating very many years of growth (possibly hundreds of years), and it is not so surprising that genetic alterations (RAPD band changes) were detected in 53% of those shoots.

It also initially appears surprising that mutation can be detected as RAPD band changes within single long shoots of *C. purpureus.* However, very few changes were actually detected—in five shoots, each analyzed in 75 sections, with 63 RAPD bands scored, only seven changes were observed in nearly 23,625 bands scored, over probably a minimum of 75 years of growth. These promising initial results suggest that Antarctic mosses might usefully be investigated as a model system for assessing mutation in a living plant. Their multi-shooted perennial colonial nature, vegetative growth, haploid genome, and division from a single meristematic cell mean that mutations can be "tested" in individual shoots without necessarily harming the rest of the colony. Similarly, single shoots can be sampled for analysis without harming the rest of the colony, enabling analysis of mutation over time. We are currently attempting to relate mutation observed within Antarctic moss shoots to growth rates and to measurements of increased levels of UV irradiation due to the ozone hole.

Antarctica holds the key to a wide range of environmental issues of a global nature, such as the effects of the ozone layer and climate change (Adamson & Adamson, 1992). It appears that investigation of genetic diversity and changes in the dominant plants—the mosses—will provide fascinating insights into some of these issues in a way not yet possible in other parts of the world.

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ORGANELLAR INHERITANCE IN THE ALLOPOLYPLOID LIVERWORT SPECIES *PORELLA BAUERI* (PORELLACEAE): RECONSTRUCTING HISTORICAL EVENTS USING DNA ANALYSIS DATA[1]

Kamila Jankowiak[2] *and Zofia Szweykowska-Kulinska*[2]

Abstract

Porella baueri (Porellaceae) is an allopolyploid species whose parent genomes are *P. cordaeana* and *P. platyphylla.* A sequence comparison of chloroplast $tRNA^{Leu}_{(UAA)}$, $tRNA^{Gly}_{(UCC)}$, and $tRNA^{Lys}_{(UUU)}$ gene introns as well as mitochondrial $tRNA^{Ser}_{(GCU)}$, *coxIII,* and *nad*5 gene introns in all three species revealed that the nucleotide sequences studied are identical in *P. baueri* and *P. cordaeana* but differ in *P. platyphylla.* Both chloroplasts and mitochondria of *P. baueri* were, therefore, inherited from one parent: *P. cordaeana.* This is the second report of uniparental transmission of organelles in liverworts, the earliest land plants. Moreover, using the $tRNA^{Leu}$ intron gene as a molecular marker, it is possible to distinguish between two morphologically similar species, *P. baueri* and *P. platyphylla.*

Key words: allopolyploidy, chloroplast genome, indels, liverworts, mitochondrial genome, nucleotide substitutions, organellar inheritance, *Porella baueri, Porella cordaeana, Porella platyphylla.*

The genus *Porella* L. (Jungermanniales, Porellaceae) includes more than 100 species of leafy, dioecious liverworts (Schuster, 1980). Of these, three species have attracted special interest: *P. platyphylla* (L.) Pfeiff., with a base chromosome number $n = 8$ (Fritsch, 1991; Paton, 1999); *P. cordaeana* (Hüb.) Moore, with $n = 8$ (Fritsch, 1991; Paton, 1999); and *P. baueri* (Schiffn.) C. Jens., with $n = 16$ (Boisselier-Dubayle et al., 1998). Boisselier-Dubayle et al. (1998) showed that *P. baueri* is an allopolyploid derivative of *P. platyphylla* and *P. cordaeana.* The geographical ranges of these three species overlap in Europe (Paton, 1999). In Poland *P. platyphylla* and *P. cordaeana* are widely distributed, whereas *P. baueri* seems to be restricted to the southern part of Poland, where it is rarely collected (Szweykowski, 1958, 2004 in press).

Identification of *Porella* taxa is difficult because of their enormous phenotypic plasticity. Using morphological traits, it is difficult to distinguish *P. baueri* from *P. platyphylla.* Boisselier-Dubayle and Bischler (1994) used a combination of molecular (i.e., isoenzyme patterns, RAPD markers) and morphological characters to delimit European taxa of *Porella.* The main conclusion of their studies was that molecular markers can be used to discriminate between species, and to reassess the morphologically distinctive characters of each taxon.

Using detailed isoenzymatic studies on *Porella cordaeana, P. platyphylla,* and *P. baueri,* Boisselier-Dubayle and Bischler (1994) established that *P. baueri* is an allopolyploid species. Moreover, they showed that hybridization between the two parental species occurred more than once. Although it is clear that an allopolyploid species like *P. baueri* has two sets of chromosomes, one derived from each parental species, the origin of chloroplast and mitochondrial genomes remains unresolved. Here we present data to show that both chloroplasts and mitochondria of *P. baueri* were consistently inherited from one parent, namely, *P. cordaeana,* and thus that organellar inheritance was uniparental.

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[1] This work is dedicated to my dear parents, Alicja Szweykowska, Polish plant physiologist, and Jerzy Szweykowski, Polish liverwort geneticist. Both died in a car accident in 2002.

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Furthermore, we show that a single DNA marker can be used to distinguish *P. platyphylla* from *P. baueri*.

Materials and Methods

SAMPLING

Herbarium specimens of *Porella* species from France, Hungary, Poland, and Spain were sampled. Voucher specimens from France and Spain are deposited in PC; voucher specimens from Hungary and Poland are deposited in POZW.

TOTAL GENOMIC DNA ISOLATION

DNA was extracted from dried herbarium material of *Porella* species using a modified version of the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, U.S.A.) procedure. One major modification was initial preparation of the dried material: 20 mg of liverwort tissue was ground in a preheated mortar (65°C) until a fine powder was obtained. Further steps of DNA isolation were as recommended in the QIAGEN protocol. The yield of isolation varied around 0.5 μg per 20 mg tissue.

POLYMERASE CHAIN REACTION (PCR)

The PCR mixture contained the following components for 10 μl reactions: 7.5 ng of total DNA; 0.5 μM primer A; 0.5 μM primer B; 1 μl 10 × buffer containing 15 mM $MgCl_2$ (QIAGEN), 1 mM spermidine (Fiedorow & Szweykowska-Kulinska, 1997); 200 μM of each dNTP; and 0.5 unit of *Taq* DNA polymerase (QIAGEN).

PCR was initiated by denaturation at 95°C for 5 min. and followed by 30 cycles: denaturation 94°C for 1 min., annealing 56°C for 1 min., elongation 72°C for 1 min., terminated by elongation at 72°C for 5 min.

Primers used for PCR reactions were based on the complete mitochondrial genome (GenBank accession number NC 001660) and chloroplast genome sequences (GenBank accession number NC 001319) from the liverwort *Marchantia polymorpha* L. (Ohyama, 1996).

Primers for PCR amplification of intron sequence of chloroplast $tRNA^{Leu}_{(UAA)}$ gene (A, B), intron sequence of chloroplast $tRNA^{Gly}_{(UCC)}$ gene (A, B), intron sequence of chloroplast $tRNA^{Lys}_{(UUU)}$ gene (A, B), intron sequence of mitochondrial $tRNA^{Ser}_{(GCU)}$ gene (A, B), and intron sequence of mitochondrial *coxIII* gene (A, B) were designed as published by us (Szweykowska-Kulinska et al., 2002). The only new designed primers for PCR amplification were these for the intron sequence of mitochondrial *nad5* gene:

A primer 5′ TGG TGC TGC TGG AAA ATC T 3′
B primer 5′ CGC CTG CTG TTA CCA TAG T 3′

Products of PCR were separated on a 0.8% agarose gel in 1 × TEB buffer and gel-isolated using QIAquick Gel Extraction Kit (QIAGEN).

CLONING AND SEQUENCING

PCR products were cloned using pGEM-T Easy Vector System (Promega, Madison, Wisconsin, U.S.A.). DNA sequencing of the direct PCR products and PCR products cloned in plasmid was performed using CEQ DTCS-Quick Start Kit and CEQ™ 2000 XL DNA Analysis System from Beckman Coulter (Fullerton, California, U.S.A.). Two populations of each liverwort species (generally one sample per each population) were always used for sequencing.

SEQUENCE DATA ANALYSIS

Alignments of nucleic acid sequences were constructed using the ClustalX program versions 1.64b and 1.81 (Thompson et al., 1997) with default settings. Irregular gaps were then modified manually. The alignments for homologous sequences have been deposited and are available in Popset (GeneBank database). Accession numbers of intron sequences deposited in GenBank database are given in Appendix 1.

RESTRICTION FRAGMENT LENGTH ANALYSIS

PCR products of chosen intron sequences were digested using the BseM II and Tsp509I enzymes. Digestion reactions were carried out according to the recommended protocols (MBI Fermentas–BseM II; New England BioLabs–Tsp509I). The digestion products were separated electrophoretically either on an 8% polyacrylamide gel (for cp $tRNA^{Gly}_{(UCC)}$ gene digests) or a 2% agarose gel (for mt *coxIII* gene, cp $tRNA^{Leu}_{(UAA)}$ digests), stained with ethidium bromide, and analyzed under a UV lamp. The restriction enzyme analysis of only 2 populations of *P. cordaeana* and *P. platyphylla* and 7 populations of *P. baueri* are shown in this paper. In the case of all other populations (9 populations of *P. cordaeana* and 12 of *P. platyphylla*) the results were identical (data not shown).

Results

To establish which parental species, *Porella platyphylla* or *P. cordaeana*, was the donor of chloro-

Table 1. The length (in bp) of liverwort introns in the chloroplast tRNA genes for leucine, glycine, and lysine; in the mitochondrial tRNA gene for serine; the first intron of the *coxIII* gene, and for the single intron of the *nad*5 gene in *Marchantia polymorpha* and three species of *Porella*.

	Intron length in organellar genes				Intron nr1 from *coxIII* gene	Intron from *nad*5 gene
	cp-$tRNA^{Leu}_{UAA}$	cp-$tRNA^{Gly}_{UCC}$	cp-$tRNA^{Lys}_{UUU}$	mt-$tRNA^{Ser}_{GCU}$		
Marchantia polymorpha	315	593	2111	991	907	672
Porella cordaeana	342	691	2110	919	886	681
P. baueri	342	691	2110	919	886	681
P. platyphylla	370	961	2110	907	883	685

plast and mitochondrial genomes to the allopolyploid species *P. baueri,* we decided to analyze six different intron sequences from both organellar genomes. Intervening sequences are known to exhibit a nucleotide substitution rate slightly lower than four-fold degenerate sites, which show the highest rate within coding sequences (Li, 1997). Consequently, in the intron nucleotide sequences derived from closely related species (such as the parental species of *P. baueri*), we expected to observe the accumulation of nucleotide substitutions and nucleotide insertions/deletions (indels). We assumed that the nucleotide sequence of analyzed organellar DNA fragments from one of the parental species should be identical, or at least much more similar, to the homologous sequence in the organellar genome of the allopolyploid species *P. baueri.* We first searched for the complete chloroplast genome sequence of the liverwort *M. polymorpha* for genes containing introns (Ohyama, 1996). We found that six chloroplast tRNA genes ($tRNA^{Leu}_{(UAA)}$, $tRNA^{Lys}_{(UUU)}$, $tRNA^{Gly}_{(UCC)}$, $tRNA^{Ile}_{(GAU)}$, $tRNA^{Ala}_{(UGC)}$, and $tRNA^{Val}_{(UAC)}$ contain introns. Furthermore, we noticed that these tRNA genes do not contain introns in the case of lower plants and Protista species (Szweykowska-Kulinska et al., 2002), for which full chloroplast genomic sequences are known, with the exception of *Chlorella vulgaris* Beij. (Day et al., 1995) and *Toxoplasma gondii* Nicoll & Manceaux (Lee et al., 2000), which contain relatively short introns in their $tRNA^{Leu}_{(UAA)}$ genes. To our satisfaction the only species of lower plant where the plastid genome contains introns within all the tRNA genes mentioned above is *Chaetosphaeridium globosum* (Nordstedt) Klebahn (Jankowiak et al., 2004). *Chaetosphaeridium globosum* belongs to Charophyta, ancestral to land plants (Karol et al., 2001). The inspection of fully sequenced chloroplast genomes of higher plants in GenBank database revealed that, in almost all cases, the six plastid tRNA genes contain introns, with the exception of *Epifagus virginiana* (L.) Bart., a parasitic species with a strong reduction of its chloroplast genome (Szweykowska-Kulinska et al., 2002). Two of the chloroplast tRNA genes that contain introns are present in two copies ($tRNA^{Ile}_{(GAU)}$ and $tRNA^{Ala}_{(UGC)}$) in the chloroplast genomes of liverworts and almost all higher plants. Our studies on organellar inheritance in the case of another allopolyploid liverwort species, *Pellia borealis* Lorber, revealed that chloroplast tRNA genes coding for leucine, glycine, and lysine also contain introns (Pacak & Szweykowska-Kulinska, 2003). These results encouraged us to use the same chloroplast intron sequences in our studies of organellar inheritance in *Porella baueri.*

The survey of fully sequenced plant mitochondrial genomes, including the liverwort *Marchantia polymorpha* (Oda et al., 1992), revealed that intron-containing tRNA genes are rare (Szweykowska et al., 2002). One tRNA gene ($tRNA^{Ser}_{(GCU)}$) in *M. polymorpha* does, however, contain an intron. Our studies of the liverwort genus *Pellia* Raddi also confirmed the presence of an intron in the mitochondrial $tRNA^{Ser}_{(GCU)}$ gene in all species studied (Pacak & Szweykowska-Kulinska, 2003). Since we have also demonstrated the presence of an intron in the mitochondrial $tRNA^{Ser}_{(GCU)}$ gene in peatmosses and mosses (unpublished data), we assumed that *Porella* species should also have this intron. In the mitochondrial genome of *M. polymorpha,* the $tRNA^{Ser}_{(GCU)}$ gene is the only tRNA gene that contains an intron. In our studies we decided to use additionally the first intron in the mitochondrial *coxIII* gene and the single intron in the mitochondrial *nad5* gene.

Based on our genomic search, we chose the following six organellar genes for sequencing: chloroplast $tRNA^{Leu}_{(UAA)}$, $tRNA^{Gly}_{(UCC)}$, and $tRNA^{Lys}_{(UUU)}$, mitochondrial $tRNA^{Ser}_{(GCU)}$, intron nr1 in *coxIII* gene, and the single intron in *nad5* gene. All of these genes are present in single copies in all fully sequenced organellar genomes, and the lengths of homologous introns in the liverworts *Marchantia po-*

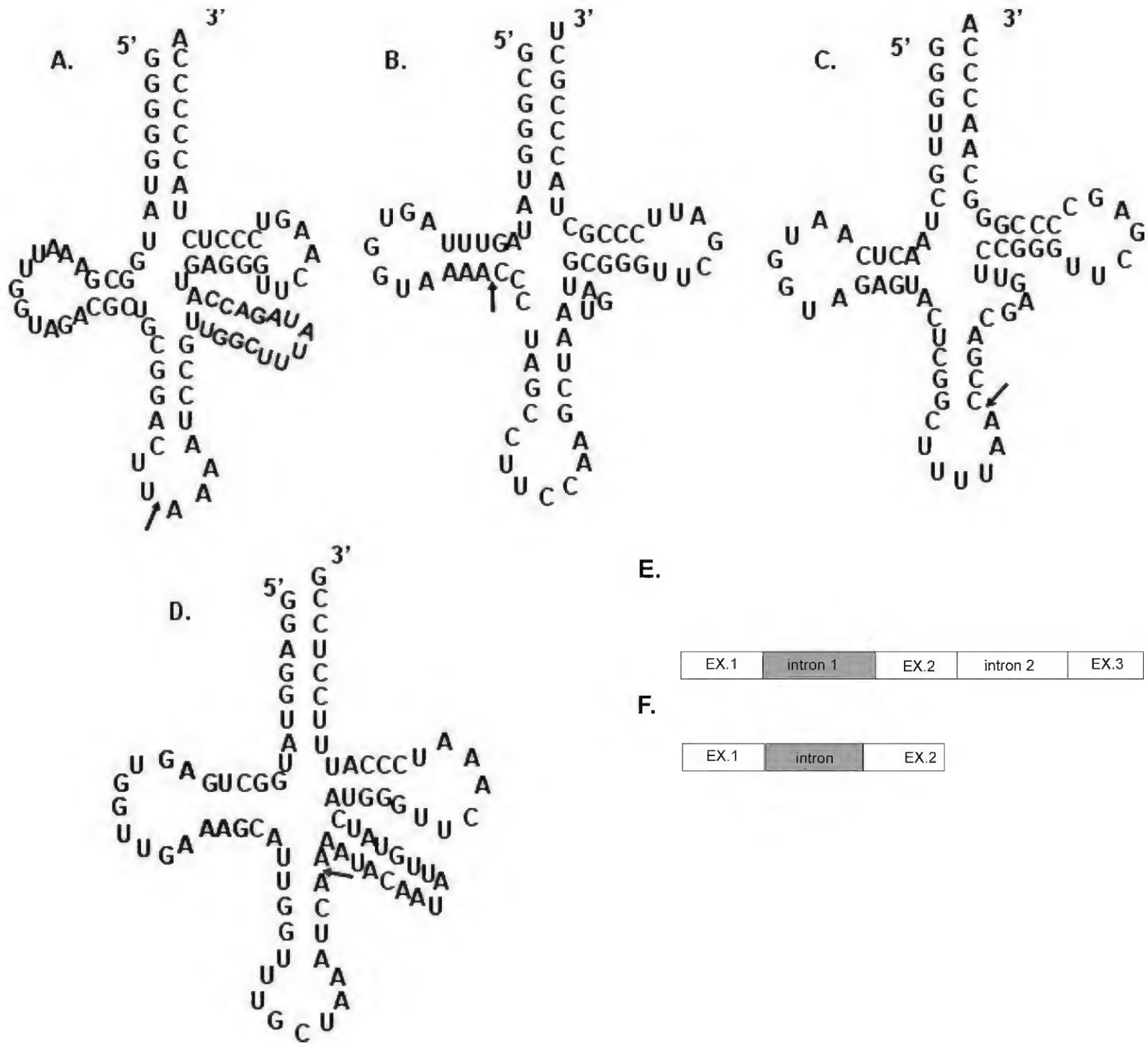

Figure 1. Intron positions in the cloverleaf structures of pre-tRNAs from the *Porella* species studied. —A. cp-$tRNA^{Leu}_{(UAA)}$. —B. cp-$tRNA^{Gly}_{(UCC)}$. —C. cp-$tRNA^{Lys}_{(UUU)}$ genes. —D. mt-$tRNA^{Ser}_{(GCU)}$ gene. —E. Exon-intron structure of the mitochondrial *coxIII* gene. —F. Exon-intron structure of the mitochondrial *nad5* gene; shaded boxes for represent introns studied.

lymorpha, *Porella cordaeana*, *P. platyphylla*, and *P. baueri* are comparable (Table 1). The intron positions in the homologous genes studied are always the same (Fig. 1).

We found that the intron sequences of chloroplast $tRNA^{Leu}_{(UAA)}$ genes from *P. cordaeana* and *P. baueri* are identical but differ from those present in *P. platyphylla*. In the latter there are three insertions and one deletion that yield a net difference in length of 28 bp. This difference is easily visible on the agarose gel electrophoretic pattern of PCR products obtained for the $tRNA^{Leu}_{(UAA)}$ gene intron from all three species (Fig. 2). *Porella platyphylla* and *P. baueri* are very difficult to discern when morphological characters are used. This simple laboratory assay is sufficient to distinguish these two species. Analysis of intron sequences from chloroplast $tRNA^{Gly}_{(UCC)}$ and $tRNA^{Lys}_{(UUU)}$ genes also revealed that *P. cordaeana* and *P. baueri* show identical sequence and that they differ from those of *P. platyphylla* by 17 and 24 nucleotide substitutions, respectively.

For mitochondrial $tRNA^{Ser}_{(GCU)}$ gene introns, we found that nucleotide sequences from *Porella cordaeana* and *P. baueri* are identical and differ from those obtained for *P. platyphylla* by two deletions. Also, in the case of the first intron of the mitochondrial *coxIII* gene and *nad5* gene, sequences in *P. cordaeana* and *P. baueri* are the same but differ from those in *P. platyphylla*. These differences are represented by one insertion and one deletion in the case of the *coxIII* gene intron and by one insertion and one substitution in the case of the *nad5* gene intron.

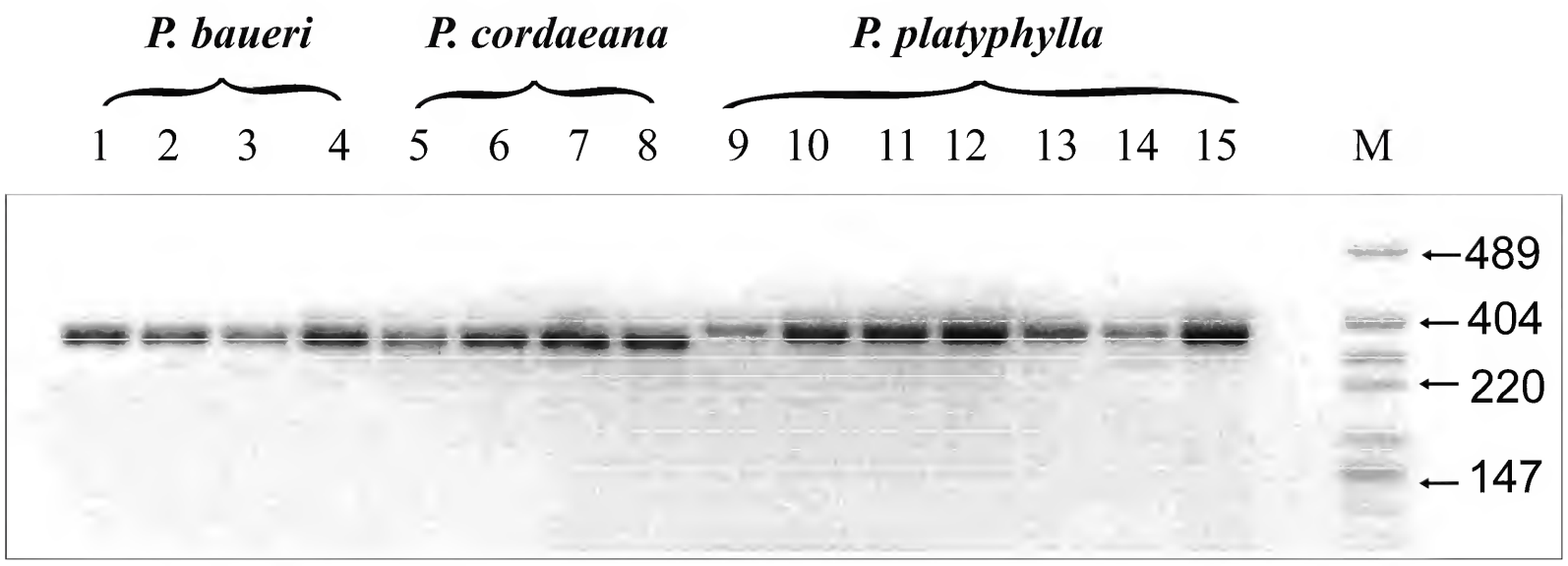

Figure 2. Electrophoretic separation on 0.8% agarose gel of PCR products derived from the amplification of chloroplast $tRNA^{Leu}_{(UAA)}$ genes. Lanes (as in gel lanes) 1–4 represent PCR products from *P. baueri*: 1. *Boisselier & Bischler 427* (PC); 2. *Bischler 1057* (PC); 3. *Bischler 1061* (PC); 4. *Mierzenska 966* (POZW). Lanes 5–8 from *P. cordaeana*: 5. *Boisselier & Bischler 289* (PC); 6. *Bischler 396* (PC); 7. *Boisselier, Bischler & Pujos 487* (PC); 8. *Bischler 1055* (PC). Lanes 9–15 from *P. platyphylla*: 9. *Mierzenska 39547* (POZW); 10. *Boisselier & Bischler 290* (PC); 11. *Boisselier 332* (PC); 12. *Bischler 335* (PC); 13. *Boisselier, Bischler & Pujos 486* (PC); 14. *Szweykowski 10584* (POZW); 15. *Szweykowski 33433* (POZW). M is marker: pGEM5Zf (+) DNA digested with MspI.

Figure 3 shows the distribution of the nucleotide sequence differences in the introns studied from *P. cordaeana* and *P. platyphylla*. Table 2 shows the number of differences in the nucleotide sequences between *P. platyphylla* and *P. cordaeana* and the Nei coefficient (Nei, 1987) for the number of nucleotide substitutions per nucleotide site.

Restriction enzymes Tsp590I and BseMII have been found to generate DNA fragments of different length for PCR products obtained for *Porella platyphylla* on one hand and *P. baueri* and *P. cordaeana* on the other. These differences enabled us to use restriction polymorphism in these fragment lengths for the analysis of other *P. cordaeana*, *P. platyphylla*, and *P. baueri* populations from Europe (France, Poland, Hungary). A total (32) of 7 populations of *P. baueri*, 14 of *P. platyphylla*, and 11 of *P. cordaeana* were studied. An example of these studies is presented in Figures 4 and 5.

Discussion

Our results clearly show that the allopolyploid liverwort *Porella baueri* inherited its chloroplast and mitochondrial genomes in a uniparental mode from *P. cordaeana*. We can not determine, however, whether this transmission was maternal or paternal. It has been found that in liverworts mitochondria and plastids are situated in the posterior part of the male gamete (There is an additional anterior mitochondrion associated with the "multilayered structure.") (Renzaglia & Garbary, 2001). We speculate that transmission of organelles occurs via female gametes since, at least in a few liverwort spe-

Table 2. Number of nucleotide substitutions, deletions, and insertions (indels) in intron sequences of the chloroplast $tRNA^{Leu}_{(UAA)}$, $tRNA^{Gly}_{(UCC)}$, $tRNA^{Lys}_{(UUU)}$ genes, mitochondrial $tRNA^{Ser}_{(GCU)}$ gene, and intron nr1 of the *coxIII* gene, and intron from *nad*5 gene in *Porella platyphylla* compared to *P. cordaeana*; and number of nucleotide substitutions per nucleotide site Nei coefficient (Nei, 1987) in sequenced chloroplast and mitochondrial genes.

	cp-$tRNA^{Leu}_{UAA}$	cp-$tRNA^{Gly}_{UCC}$	cp-$tRNA^{Lys}_{UUU}$	mt-$tRNA^{Ser}_{GCU}$	Intron nr1 from *coxIII* gene	Intron from *nad*5 gene
Deletions	1	0	0	2	1	0
Insertions	3	0	0	0	1	1
Indels	4	0	0	2	2	1
Substitutions	2	17	24	0	0	1
			Nei coefficient			
	5.61×10^{-3}	24.60×10^{-3} $\approx 13.86 \times 10^{-3}$	11.37×10^{-3}	0	0	1.46×10^{-3} $\approx 1.46 \times 10^{-3}$

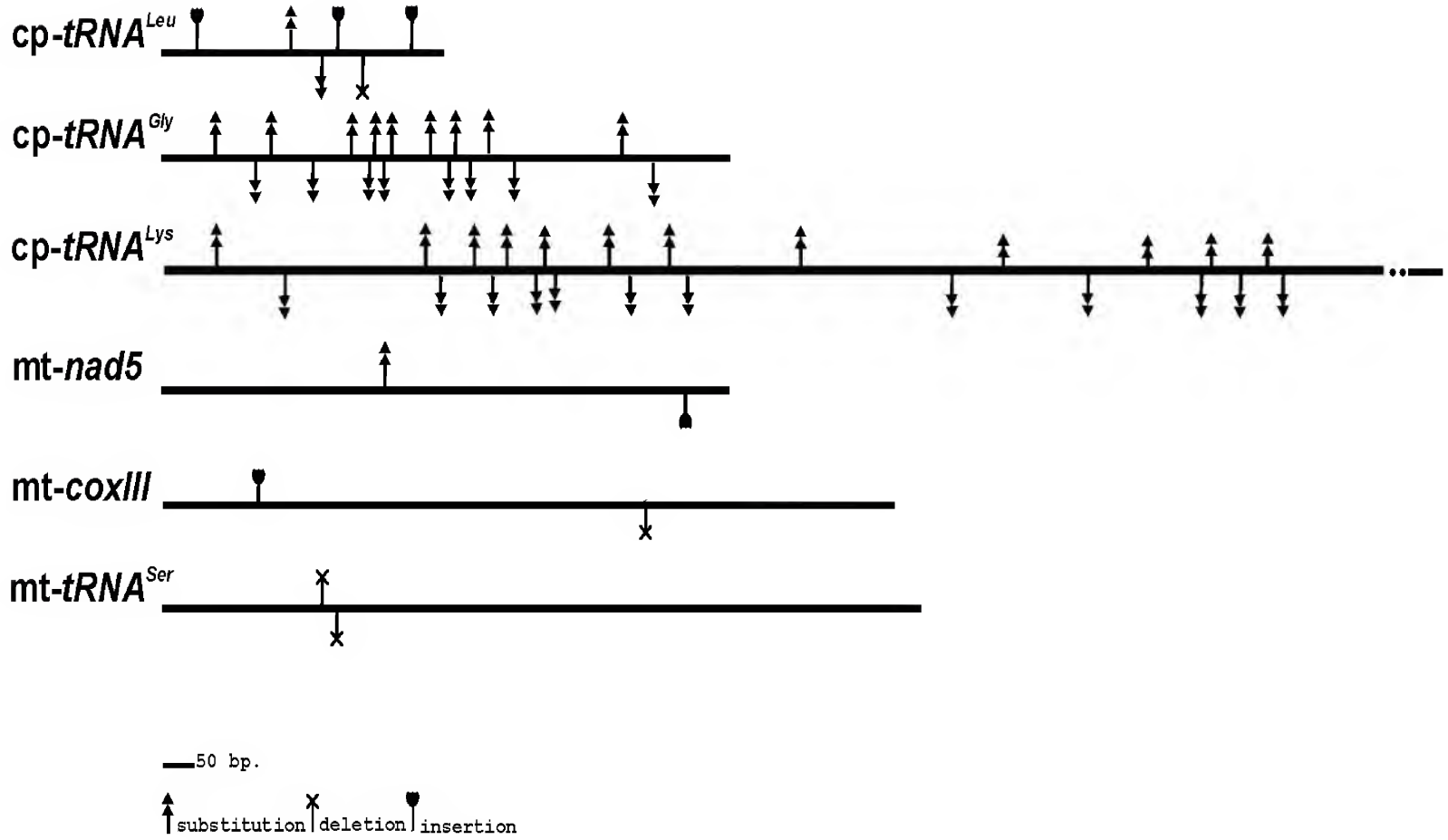

Figure 3. Diagram showing the distribution of the differences in the intron nucleotide sequences of the chloroplast $tRNA^{Leu}_{(UAA)}$; $tRNA^{Gly}_{(UCC)}$; $tRNA^{Lys}_{(UUU)}$ and mitochondrial *nad5, coxIII,* $tRNA^{Ser}_{(GCU)}$ genes in *Porella platyphylla* and *P. cordaeana.* The drawing is approximately to scale.

cies, the posterior part of the male gamete is rejected before fertilization (Duckett et al., 1983; Renzaglia & Garbary, 2001).

Boisselier-Dubayle et al. (1998) discovered differences in the isoenzyme patterns between western and eastern European populations of *Porella baueri.* The authors suggested that interspecific hybridization could take place more than once. In our studies on organellar inheritance, we investigated western (France) and eastern (Hungary, Poland) European populations of *P. baueri.* In all of these samples *P. cordaeana* was the donor of chloroplasts and mitochondria to *P. baueri.* If the hypothesis of multiple (at least two) interspecific hybridization events is true, as suggested by analysis of loci in the nuclear genome, *P. cordaeana* always transmitted its organelles to the derivatives, at least in the populations studied. Considering the above assumptions, it is probable that *P. cordaeana* was, in all hybridization events, the female parent. Figure 6 summarizes the organellar inheritance in the case of *P. baueri.*

Pellia borealis is an allopolyploid liverwort whose parents are two sibling species, *Pellia epiphylla* (L.) Corda–species N and *Pellia epiphylla*–species S. Three haplotypes of *P. borealis* have been described in Europe suggesting at least three interspecific hybridization events in the past (Odrzykoski et al., 1996). Preliminary studies suggest that these haplotypes, like *Porella baueri* haplotypes, exhibit distinct west-east geographical distribution (Odrzykoski et al., 1996). In our previous studies on organellar inheritance in *P. borealis,* we have shown that in all populations representing the three haplotypes of this allopolyploid species *P. epiphylla*–species N was always the donor of organelles (Pacak & Szweykowska-Kulinska, 2003). This observation would suggest that in all hybridization events the same parental species always transmitted its organelles to the derivative. These results in *Pellia borealis* strongly resemble our data for *Porella baueri.*

Pellia borealis and *Porella baueri* represent the only two known examples of allopolyploid liverwort species with well-documented parental species and in which origin of chloroplasts and mitochondria has been determined. In both cases we observed uniparental inheritance of organelles. Organellar transmission has been studied using cytological and genetic approaches in many higher plants. The results of these studies reveal different patterns of organellar inheritance. In angiosperms the main model is maternal uniparental (Rajora et al., 1992; Gillham, 1994; Small & Wendel, 1999). Exceptions, such as *Oenothera* L., *Pelargonium* L'Her. ex Aiton, and *Medicago* L., in which biparental transmission of plastids and maternal inheritance of mitochondria have been reported (Mogensen, 1996).

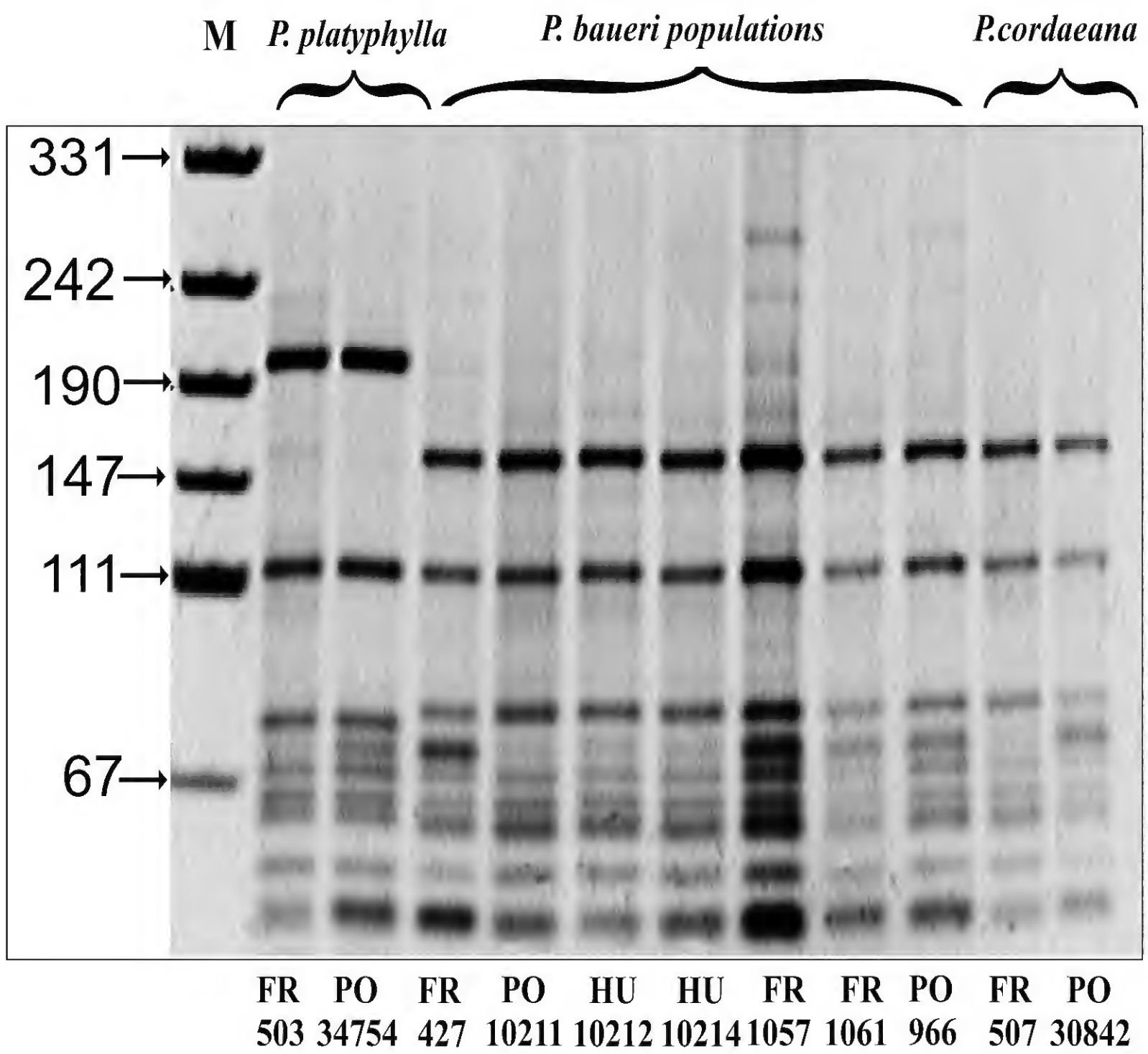

Figure 4. Electrophoretic separation on an 8% polyacrylamide gel of PCR products derived after amplification of chloroplast $tRNA^{Gly}_{(UCC)}$ intron genes digested with Tsp509I. M is marker: pUC 19 DNA digested with Msp I. Gel Lanes: 1. *Porella platyphylla Bischler 503* (PC); 2. *Porella platyphylla Szweykowski 34754* (POZW). Lanes 3–9, different *P. baueri* populations: 3. *Boisselier & Bischler 427* (PC); 4. *Szweykowski 10211* (POZW); 5. *Póes 10212* (POZW); 6. *Póes 10214* (POZW); 7. *Bischler 1057* (PC); 8. *Bischler 1061* (PC); 9. *Mierzenska 966* (POZW). Lanes: 10. *Porella cordaeana Boisselier, Bischler & Pujos 507* (PC); 11. *Porella cordaeana Szweykowski 30842* (POZW).

In gymnosperms the general pattern is uniparental, maternal except in conifers, which are uniparental, paternal except for Pinaceae and Taxaceae, in which maternal inheritance of mitochondria has been shown (Dong & Wagner, 1994; Mogensen, 1996). The reports concerning organellar transmission in ferns describe a uniparental, maternal pattern (Kuroiwa, 1991). There are no reports, besides our own, concerning plastid and mitochondria inheritance in Bryophyta. In algae that produce isogametes, the process is uniparental, and in the case of anisogamous and oogamous algae, it is uniparental and maternal (Law & Huston, 1992; Gillham, 1994; Birky, 1995, 2001). From this short review it can be concluded that the major type of organellar transmission in plants appears uniparental. Our reports concerning *Pellia borealis* and *Porella baueri* agree with this conclusion.

Comparison of the number of nucleotide substitutions per site in the case of chloroplast and mitochondrial intron sequences in *P. platyphylla* and *P. cordaeana* shows that this value is eight times higher in chloroplasts than in mitochondria. Similar studies on the rate of nucleotide substitution within intron sequences in *Pellia borealis–P. epiphylla*–species N and *Pellia epiphylla*–species S revealed that this rate is six times higher in plastids than in mitochondria (Pacak & Szweykowska-Kulinska, 2003). In angiosperms it has been postulated that cpDNA evolves at least three to four times faster

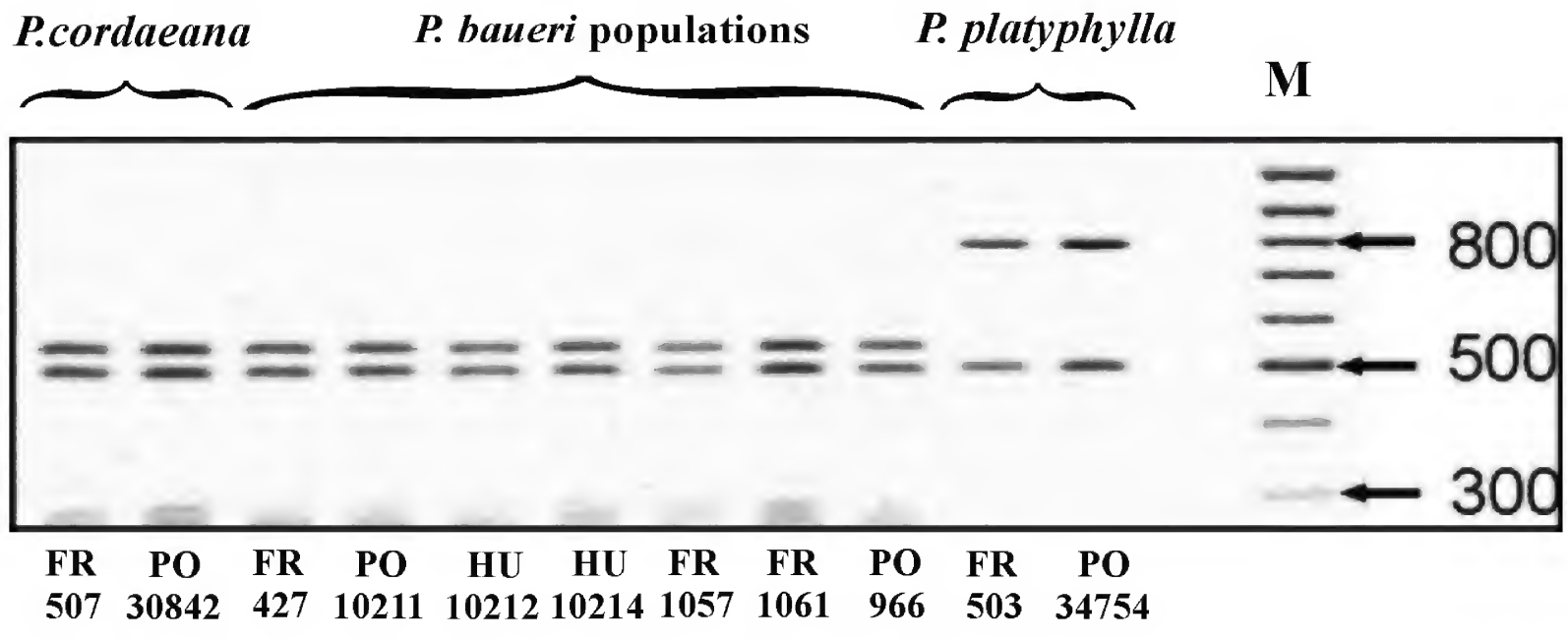

Figure 5. Electrophoretic separation on a 2% agarose gel of the PCR product derived after amplification of the part of mitochondrial *coxIII* gene containing intron 1 digested with BseM II. M is DNA molecular weight marker Gene Ruler 100-bp DNA Ladder. Gel Lanes: 1. *Porella platyphylla Bischler 503* (PC); 2. *Porella platyphylla Szweykowski 34754* (POZW). Lanes 3–9 different *P. baueri* populations: 3. *Boisselier & Bischler 427* (PC); 4. *Szweykowski 10211* (POZW); 5. *Póes 10212* (POZW); 6. *Póes 10214* (POZW); 7. *Bischler 1057* (PC); 8. *Bischler 1061* (PC); 9. *Mierzenska 966* (POZW). Lanes: 10. *Porella cordaeana Boisselier, Bischler & Pujos 507* (PC); 11. *Porella cordaeana Szweykowski 30842* (POZW).

than mtDNA (Wolfe et al. 1987; Palmer & Herbon, 1988; Palmer, 1990). Our estimates of the nucleotide substitution rate in liverworts suggest a two-fold higher value than in angiosperms. When particular chloroplast intron genes from *Porella platyphylla* and *P. cordaeana* are compared, the number of nucleotide substitutions per nucleotide site varies considerably from 5.61×10^{-3} in the case of the cp$tRNA^{Leu}_{(UAA)}$ gene to 24.60×10^{-3} in the case of the cp$tRNA^{Gly}_{(UCC)}$ gene. Generally, these values are higher than in *Pellia* species (Pacak & Szweykowska-Kulinska, 2003). This suggests that *Porella platyphylla* and *P. cordaeana* diverged from each other earlier than *Pellia epiphylla*–species N and *Pellia epiphylla*–species S. The number of indels is lower, however, in plastid (4 indels) than in mitochondrial (5 indels) DNA sequences in *Porella*. In *Pellia* this difference is much greater: 4 indels in chloroplast introns and 17 indels in mitochondrial (Pacak & Szweykowska-Kulinska, 2003). Although the tendency seems to be the same (more indels in mitochondrial DNA than in chloroplast DNA), the mitochondrial $tRNA^{Ser}_{(GCU)}$ intron in *Pellia* species accumulated more DNA rearrangements than the homologous intron in *Porella*. This may indicate that *Pellia* mitochondrial DNA is more susceptible to insertion/deletion mutations and reopens the question about an earlier origin of *Porella baueri* versus *Pellia borealis*. The higher number of indels in mtDNA than in cpDNA in liverworts

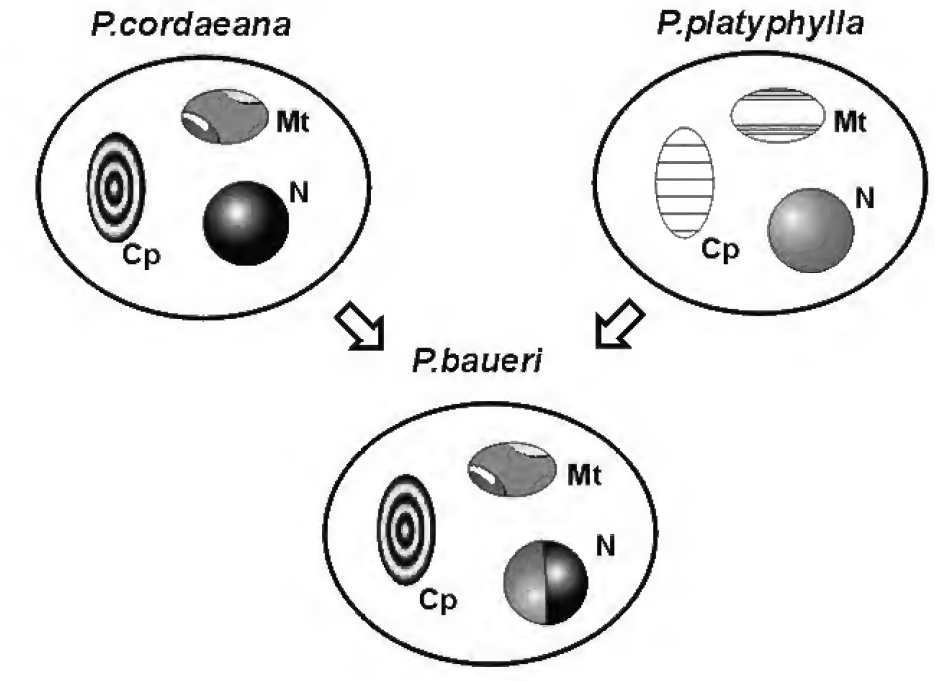

Figure 6. The origin of chloroplast and mitochondria in the allopolyploid species *Porella baueri*.

agrees with data obtained for angiosperms, which shows that mitochondrial genomes evolve rapidly in structure but slowly in sequence (Palmer & Herbon, 1988).

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Appendix 1. Voucher specimens and GenBank accession numbers utilized in this study.

Taxon	Collection	GenBank accession No.					
		intron of cp-*tRNA*$^{Leu}_{(UAA)}$	intron of cp-*tRNA*$^{Gly}_{(UCC)}$	intron of cp-*tRNA*$^{Lys}_{(UUU)}$	intron of mt-*tRNA*$^{Ser}_{(GCU)}$	intron nr1 from *coxIII* gene	intron from *nad*5 gene
Porella baueri (Schiffn.) C.E. O. Jensen	France, Bretagne: Morbihan, *Boisselier & Bischler 427* (PC)	—	—	—	AY245580	—	—
Porella baueri	France. Bretagne: Monument aux Morts, *Bischler 1057* (PC)	—	—	AY168653	—	—	AY278029
Porella baueri	France. Bretagne: école St. Hervé, *Bischler 1061* (PC)	AF462148	AY168650	—	AY245580	AY245583	—
Porella baueri	Poland. Bieszczady Mts.: Tarnica, *Mierzenska 966* (POZW)	AF46148	AY168650	AY168653	—	—	AY278029
Porella baueri	Poland. Bieszczady Mts.: Przyslup, *Szweykowski 10211* (POZW)	—	—	—	—	AY245583	—
Porella baueri	Hungary. Matra Mts.: *Póes 10212, 10214* (POZW)	—	—	—	—	—	—
Porella cordaena (Huebener) Moore	France. Bretagne: Rochefort en Terre, *Bischler 1055* (PC)	—	—	—	—	—	—
Porella cordaeana	France. Jura: Franche-Comte, *Boisselier & Bischler 289* (PC)	—	—	—	—	—	—
Porella cordaeana	France. Centre: Eure & Loire, *Bischler 396* (PC)	—	—	AY168654	—	—	—
Porella cordaeana	France. Languedoc-Roussillon: Herault vers Castanet le Haut, *Boisselier, Bischler & Pujos 487* (PC)	—	—	—	—	—	—
Porella cordaeana	France. Languedoc-Roussillon: Aude, Cals, *Boisselier, Bischler & Pujos 507* (PC)	AF462149	AY168651	—	AY245581	AY245584	AY278030
Porella cordaeana	Poland. Debno Lake: *Szweykowski, Buczkowska & Jankowiak 30842, 39707, 39709, 39710* (POZW)	AF462149	AY168651	AY168654	AY245581	AY245584	AY278030
Porella cordaeana	Poland. Tatra Mts.: Dolna Mietusia, Wielka Swistówka, *Szweykowski 37057* (POZW)	—	—	—	—	—	—
Porella cordaeana	Poland. Tatra Mts.: Trzydniowianski Wierch SW, *Szweykowski 39523* (POZW).	—	—	—	—	—	—
Porella platyphylla (L.) Pfeiff.	Poland. Zimny Dól: near Krakow, *Mierzenska 39547* (POZW)	—	—	—	—	—	—
Porella platyphylla	Poland. Upland Czestochowska: Zawiercie, *Stebel 39546* (POZW)	—	—	—	—	—	—
Porella platyphylla	Poland. Upland Czestochowska: Berkowa Mt., Korczyce, *Szweykowski 39524* (POZW)	—	—	—	—	—	—
Porella platyphylla	Poland. Bieszczady Mts.: Suche Rzeki, *Mierzenska 39741* (POZW)	—	—	—	—	—	—
Porella platyphylla	Poland. Biezczady Mts.: Pszczeliny, *Szweykowski 34754* (POZW)	AY007637*	AY168652	AY168655	AY245582	AY245585	AY278031
Porella platyphylla	Poland. Bieszczady Mts.: Pasmo Otrytu, Hulskie, *Szweykowski 33433* (POZW)	—	—	—	—	—	—

Appendix 1. Continued.

Taxon	Collection	GenBank accession No.					
		intron of cp-$tRNA^{Leu}_{UAA}$	intron of cp-$tRNA^{Gly}_{UCC}$	intron of cp-$tRNA^{Lys}_{UUU}$	intron of mt-$tRNA^{Ser}_{GCU}$	intron nr1 from *coxIII* gene	intron from *nad*5 gene
Porella platyphylla	Poland. Kaczawskie Mts.: Okole, *Szweykowski, Buczkowska & Jankowiak 39698* (POZW)	—	—	—	—	—	—
Porella platyphylla	Poland. Kaczawskie Mts.: Ostrzyca, *Szweykowski, Buczkowska & Jankowiak 39702* (POZW)	—	—	—	—	—	—
Porella platyphylla	Poland. Pieniny Mts.: Pieninski Potok, *Szweykowski 10584* (POZW)	—	—	—	—	—	—
Porella platyphylla	France. Midi-Pyrenees: Tarn, *Boisselier, Bischler & Pujos 486* (PC)	—	—	—	—	—	—
Porella platyphylla	France. Jura: Franche-Comte, *Boisselier & Bischler 290* (PC)	—	—	—	—	—	—
Porella platyphylla	France. Centre: Loir & Cher, *Boisselier 332* (PC)	—	—	—	—	—	—
Porella platyphylla	France. Languedoc-Roussillon: Herault, *Boisselier, Bischler & Pujos 503* (PC)	AY007637*	AY168652	AY168655	AY245582	AY245585	AY278031
Porella platyphylla	Spain. Navarra: Col d'Ibaneta, *Bischler 335* (PC)	—	—	—	—	—	—

* Sequences were previously deposited in GenBank database by M. Stech & W. Frey from Freie Universitaet Berlin, Germany (Stech & Frey, 2001). Nucleotide sequences obtained by us were identical.

MOLECULAR RELATIONSHIPS AND BIOGEOGRAPHY OF TWO GONDWANAN *CAMPYLOPUS* SPECIES, *C. PILIFER* AND *C. INTROFLEXUS* (DICRANACEAE)[1]

Michael Stech[2] *and Joana Dohrmann*[2]

Abstract

Campylopus pilifer and *C. introflexus* are two closely related species probably of Gondwanan origin. To provide molecular characters for a clearer delimitation of both species, and to analyze infraspecific divergence and biogeographical patterns, sequences of the nuclear ribosomal DNA ITS1 and ITS2 regions as well as the chloroplast DNA *atp*B-*rbc*L spacer region of specimens covering most parts of the respective species distribution areas are analyzed under the criteria of maximum parsimony and maximum likelihood. The molecular data resolve *C. introflexus* as a monophyletic taxon with only low infraspecific sequence variation, making it impossible to determine the origin of the populations introduced into North America and Europe. In contrast, *C. pilifer* is split into an Old World lineage (Africa and Arabia) and one or two New World lineages (the Americas), which probably result from divergent evolution after the separation of the former Gondwana supercontinent. Populations of *C. pilifer* from the Macaronesian archipelagos and continental Europe also belong to the Old World group. Further geographical differentiation at the molecular level is observed mainly in the American *C. pilifer,* and examples of possible long-range dispersal or introduction also exist. While the ITS2 region is, despite high sequence variation, comparable in all *Campylopus* species analyzed so far, two very different types of the ITS1 region occur, one in the African group of *C. pilifer* and in *C. introflexus,* the second in the American group of *C. pilifer* and in other *Campylopus* species. Possible reasons for this pattern are discussed.

Key words: *atp*B-*rbc*L spacer, biogeography, *Campylopus introflexus, Campylopus pilifer,* ITS, molecular relationships.

The moss genus *Campylopus* Brid. is probably of Gondwanan origin (Frahm, 1988). Of the approximately 150 species (Frahm, 1999, 2001), about 15 still show Gondwanan or subantarctic distribution patterns (Frahm, 1988). Five of them, *Campylopus flexuosus* (Hedw.) Brid., *C. fragilis* (Brid.) Bruch & Schimp., *C. introflexus* (Hedw.) Brid., *C. pilifer* Brid., and *C. pyriformis* (Schultz) Brid., extended their distribution area into the Northern Hemisphere. The Northern Hemispheric populations are either regarded as relics of warmer climate periods, or the respective species represent neophytes of the North American and European floras (Frahm, 1984, 1988, 2001).

Campylopus pilifer occurs today from South America to North America, Africa, Arabia, India, Sri Lanka, the Macaronesian archipelagos, western Europe, and the Mediterranean region, whereas the subantarctic *C. introflexus* shows a bipolar disjunction (southern South America, southern Africa, parts of Australia, New Zealand, subantarctic islands, introduced into Europe and eastern North America in the 20th century; Frahm, 1988) (Fig. 1).

Both species are closely related morphologically and anatomically and may be difficult to distinguish, especially when sterile. In fact, *Campylopus pilifer* and *C. introflexus* were considered synonymous from Mitten (1869) onward until the detailed study of Giacomini (1955) was published, and it took another decade to finally achieve reliable systematic and biogeographical circumscriptions of both species (Richards, 1963; Barkman & Mabelis, 1968; Frahm, 1974, 1975; Gradstein & Sipman, 1978). Typical plants of *C. pilifer* and *C. introflexus* differ from each other, e.g., by the hyaline hairpoints at the leaf tips (erect vs. reflexed about 90° in dry condition), the height of the lamellae on the

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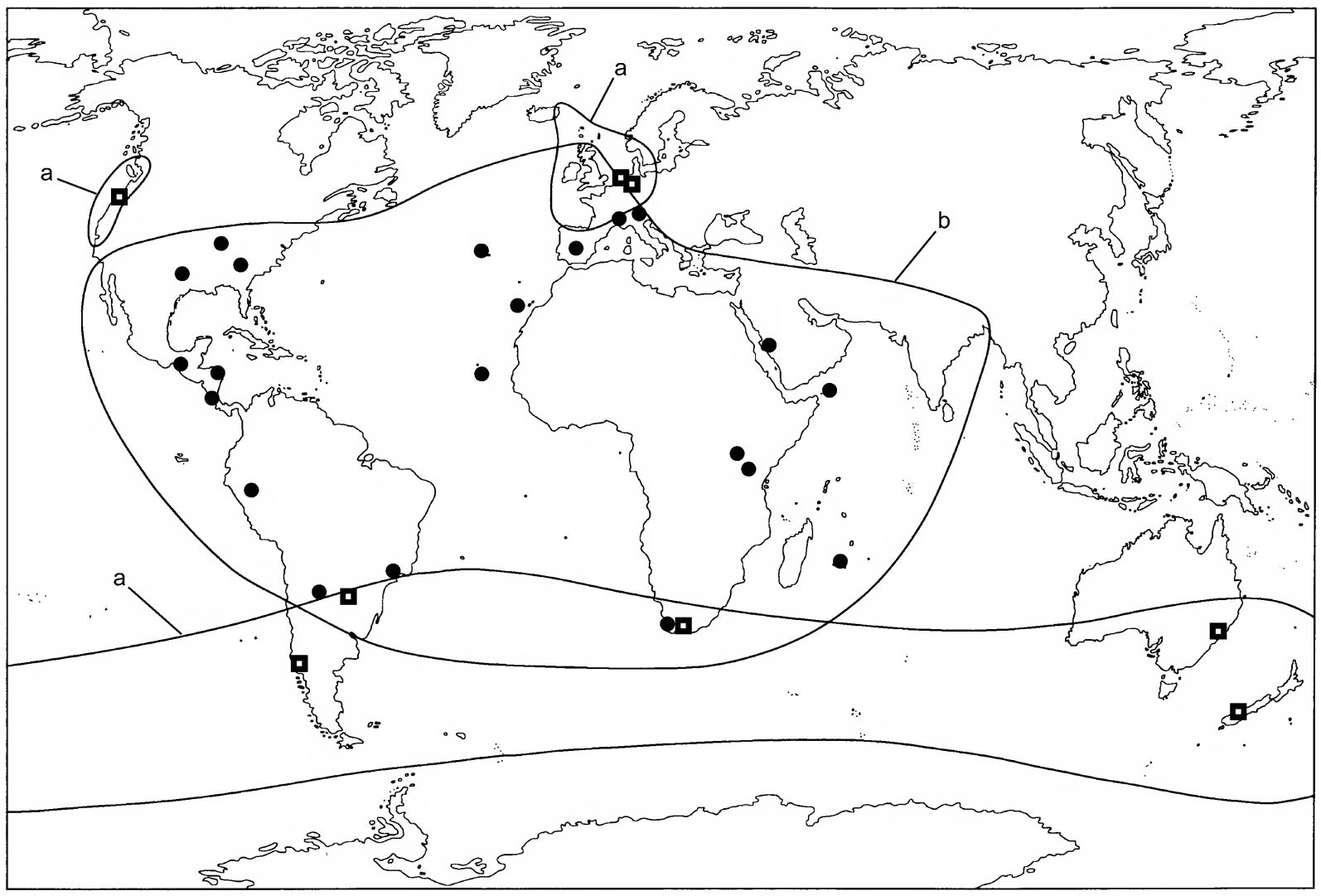


Figure 1. Distribution map of *Campylopus introflexus* (a) and *C. pilifer* (b), showing the geographical origin of the specimens used for sequencing (*C. introflexus*: open squares, *C. pilifer*: dots). The lines demarcate the natural distribution of both species according to Frahm (1985, 2001).

dorsal surface of the costa (2 to 7 vs. 1 to 2 cells), and the length of the seta (3–5.5 vs. 5–12 cm, respectively). The latter character, however, is often difficult to use because *C. pilifer* rarely produces sporophytes. In addition, hybridization between both species was assumed based on the high percentages of aborted spores in unopened capsules of European and African *C. pilifer* (Gradstein & Sipman, 1978) and plants morphologically intermediate between both species (Frahm, 1985, 1988).

An initial molecular study concerning the circumscription and infrageneric relationships of *Campylopus* (Stech, 2004) revealed a high level of sequence variation in the ITS1 and ITS2 regions of the nuclear ribosomal (nr) DNA, and only little variation in chloroplast (cp) DNA markers (*trn*T-L spacer and *trn*L intron of the *trn*T-F region, *atp*B-*rbc*L spacer). However, the suitability of these markers to differentiate between *Campylopus pilifer* and *C. introflexus* could not be evaluated, as no specimen of *C. introflexus* was included. Therefore, the nrDNA ITS1 and ITS2 regions and the cpDNA *atp*B-*rbc*L spacer were sequenced for a larger number of samples for these two species (31 and 12 accessions, respectively), covering most parts of their respective distribution areas (Fig. 1). The nrDNA ITS region is one of the most frequently used molecular markers in angiosperms as well as in bryophytes (Quandt & Stech, 2003) and has also been explored in population level studies of bryophytes (e.g., Chiang & Schaal, 1999; Shaw, 2000; Shaw & Allen, 2000; Stech et al., 2001; Schaumann et al., 2003; Shaw et al., 2003; Werner et al., 2003). The *atp*B-*rbc*L spacer was chosen for comparison with the ITS tree topologies with respect to its higher variability in *Campylopus* compared to the *trn*T-F region (Stech, 2004). The aims of this study are (1) to test the monophyly of *Campylopus pilifer* and *C. introflexus* and to provide molecular characters for a clearer delimitation of both species, and (2) to reveal possible infraspecific sequence divergence and to evaluate if this divergence shows distinct biogeographical patterns.

Materials and Methods

Plant material

Sequences of the ITS1/5.8S rDNA/ITS2 region were determined from 30 specimens of *C. pilifer* var. *pilifer*, 1 specimen of *C. pilifer* var. *lamellatus*, and 12 specimens of *C. introflexus*, while the *atp*B-*rbc*L spacer was sequenced in 8 specimens of *C.*

pilifer (including the specimen belonging to var. *lamellatus*) and 5 specimens of *C. introflexus* (Table 1). For comparison, 22 sequences of 20 other *Campylopus* species and representatives of the genera *Dicranodontium* Bruch & Schimp., *Leucobryum* Hampe, and *Pilopogon* Brid. already analyzed in Stech (2004) were included. All plant material was prepared for DNA extraction through cleaning with water and ultrasonic treatment. Voucher specimens are deposited in BONN.

DNA EXTRACTION, PCR, AND SEQUENCING REACTIONS

DNA preparations were carried out following the method of Doyle and Doyle (1990). PCR reactions (50 µl) were performed in a Biometra thermocycler and contained 1.7 U *Taq* DNA polymerase (QIAGEN), 1× buffer, 1.5 mM $MgCl_2$, 1mM dNTP-mix of each 0.25 mM dNTPs (Roth), and 10 pmol of primers 18F/25R (after White et al., 1990) for ITS1/5.8S/ITS2 and atpB-1/rbcL-1 (Chiang et al., 1998) for the *atp*B-*rbc*L spacer. Cycling protocols were: 5 min. at 94°C, 35 cycles (45 sec. at 94°C, 45 sec. at 48°C, 1 min. at 72°C), 4 min. at 72°C for ITS; and 2 min. at 94°C, 30 cycles (45 sec. At 94°C, 1 min. 15 sec. at 49°C, 1 min. 15 sec. at 72°C), 10 min. at 72° for *atp*B-*rbc*L spacer.

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN). Cycle sequencing reactions (2 min. at 94°C, 35 cycles [30 sec. at 94°C, 30 sec. at 60°C, 30 sec. at 72°C], 1 min. at 72°C) were carried out in a Perkin Elmer thermocycler using the SequiTherm Excel II DNA Sequencing Kit (Epicentre) and 5′ biotin-modified primers (Roth). In addition to the amplification primers, ITS primers 5.8F/5.8R (after White et al., 1990) and two new primers designed to match a conserved region of the ITS2 in *Campylopus* (iF: 5′-GAG TGG AAC TGG CCA TCC-3′, iR: 5′-GGA TGG CCA GTT CCA CTC-3′) were used to determine GC-rich regions in the ITS1 and ITS2 regions. Sequencing reactions were separated in the GATC-1500-system, transferred to Nylon membranes (Pall) and visualized applying a standard protocol with Streptavidin-Alkaline Phosphatase (Promega) and BCIP/NBT (Roth) treatment. Sequences were determined manually from the membranes and deposited in GenBank under the accession numbers listed in Table 1.

ALIGNMENT AND TREE CONSTRUCTION

Alignments of the sequences were made manually in the Alignment Editor Align32 (Hepperle, 1997). Maximum parsimony and maximum likelihood trees were evaluated with PAUP* 4.0b10 (Swofford, 2002). Heuristic parsimony searches of five data sets (ITS1, ITS2, combined ITS1 and ITS2, *atp*B-*rbc*L spacer, and combined nrDNA and cpDNA) were performed with the following options: all characters unweighted and unordered, multistate characters interpreted as uncertainties, gaps coded as missing data, performing TBR branch swapping, collapse zero length branches, MulTrees option in effect, random addition sequence with 1000 replicates, and maxtrees = 100,000. *Dicranodontium* and *Leucobryum* were chosen as outgroup representatives in the cpDNA analyses to confirm the sister group relationship of *Campylopus* and *Pilopogon* indicated in Stech (2004). In the ITS and combined nrDNA + cpDNA analyses, *Pilopogon* was used as outgroup representative for *Campylopus* as the sequences of the other genera were hardly alignable, especially in the ITS1 region. Heuristic bootstrap searches were performed with 1000 replicates with 10 addition sequence replicates per bootstrap replicate. Further measurement of support for individual clades was obtained using AutoDecay 4.0 with 100 random addition cycles (Eriksson, 1999). Maximum likelihood calculations were executed assuming a GTR + G + I model for ITS and combined nrDNA + cpDNA analyses, or GTR + G model for *atp*B-*rbc*L spacer, respectively, and a rate variation among sites following a gamma distribution (four categories each represented by a mean average rate). GTR + G + I or GTR + G were chosen as the models that best fit the data under the AIC criterion as evaluated by Modeltest v.4b (Posada & Crandall, 1998) employing the Windows front-end (Patti, 2002). The settings proposed by Modeltest were: Basefreq = (0.2762 0.2559 0.2340), Nst = 6, Rmat = (1.6327 3.4654 0.8048 2.5342 5.5927), Shape = 0.6740, Pinvar = 0.2547 for ITS; Basefreq = (0.4355 0.0780 0.0995), Nst = 6, Rmat = (1.0000 5.5723 0.2301 0.2301 5.5723), Shape = 0.3872, Pinvar = 0 for *atp*B-*rbc*L spacer; and Basefreq = (0.3433 0.1827 0.1772), Nst = 6, Rmat = (1.6927 4.0097 0.5904 4.5983 6.9363), Shape = 0.5805, Pinvar = 0.4481 for combined ITS + *atp*B-*rbc*L spacer. Likelihood bootstrap searches were performed with 100 replicates using the "fast bootstrap" option (bootstrapping without branch-swapping) of PAUP* (Swofford, 2002). Fast bootstrapping was shown to provide estimates of support similar to, although generally less than, bootstrapping with branch-swapping, and allows to achieve a reasonable computation time (Mort et al., 2000).

Table 1. Voucher information and GenBank Accession numbers for the sequences used in this study. Asterisks indicate accessions not previously analyzed in Stech (2004). Missing data is indicated by —. All herbarium vouchers are deposited at BONN.

Taxon	Geographical origin	Voucher	GenBank accession No.	
			ITS1/5.8S/ITS2	*atp*B-*rbc*L spacer
INGROUP				
Campylopus acuminatus var. *kirkii* (Mitt.) J.-P. Frahm	New Zealand	26 Feb. 1998, *Frahm s.n.*	AY540857	AY159934
C. anderssonii (Müll. Hal.) A. Jaeger	Bolivia	*Lewis 88-305*	AF444138	AY159923
C. australis Catchs. & J.-P. Frahm 1	Australia	*Streimann 56175*	AF444129	AY159925
C. australis 2	Australia	*Blackwell 38.92*	AY504854	AY159924
C. bicolor (Hornsch. ex Müll. Hal.) Wilson	South Africa	*Arts RSA129/30*	AF444133	AY159933
C. crateris Besch.	Réunion	*Müller R638*	AF444137	AY159939
C. cryptopodioides Broth.	Brazil	*Schäfer-Verwimp & Verwimp 12018*	AF444120	AY159915
C. dichrostris (Müll. Hal.) Paris	Brazil	*Schäfer-Verwimp 6807*	AF444124	AY159910
C. exasperatus var. *lorentzii* (M. Fleisch.) J.-P. Frahm	Philippines	*Schwarz 3820*	AF444131	AY159929
C. flexuosus (Hedw.) Brid.	Germany	*Stech B960905.2*	AF444117	AY159919
C. fragilis (Brid.) Bruch & Schimp.	Australia	*Streimann 51759*	AF444122	AY159917
C. gracilis (Mitt.) A. Jaeger	Sri Lanka	*Schäfer-Verwimp 5430/1*	AY540859	AY159938
C. hensii Renault & Cardot	Equatorial Guinea	*Heras 25.7.94,* dupl. ex *VIT443/94*	AF444126	AY159911
**C. introflexus* (Hedw.) Brid. 1	Australia	*Coveny & P. D. Hind 15865*	AY375229	AY551363
**C. introflexus* 2	New Zealand	*Frahm X10-1*	AY375231	—
**C. introflexus* 3	Chile	*Schäfer-Verwimp & Verwimp 8073*	AY375223	—
**C. introflexus* 4	Chile	*Frahm 12-1*	AY375224	AY551365
**C. introflexus* 5	Paraguay	*Zardini & Velásquez 26060*	AY375222	AY576441
**C. introflexus* 6	U.S.A., Oregon	*Raeymaekers 5143*	AY375227	AY551366
**C. introflexus* 7	South Africa	*van Zanten et al. 760873*	AY375230	—
**C. introflexus* 8	South Africa	*Hedderson 13682*	AY574223	—
**C. introflexus* 9	Germany	16 Feb. 1997, *Frahm s.n.*	AY375226	—
**C. introflexus* 10	Germany	*Stech B890407.1*	AY375225	AY551367
**C. introflexus* 11	Netherlands	*van Zanten 01.08.01B*	AY375236	—
**C. introflexus* 12	Netherlands	*van Zanten 01.08.01A*	AY375228	—
C. julicaulis Broth.	Paraguay	*Zardini 6652*	AF444119	AY159914
C. macgregorii Broth. & Geh.	Malaysia	30 Aug. 1986, *Frahm s.n.*	AF444128	AY541682
**C. pilifer* Brid. 1	South Africa	*Matcham 4153a*	AY372471	—
**C. pilifer* 2	Réunion	*Pfeiffer 2002-27*	AY375218	—
**C. pilifer* 3	Réunion	*Pfeiffer 2002-32*	AY375219	—
**C. pilifer* 4	Réunion	*Pfeiffer 2002-30b*	AY375217	—
**C. pilifer* 5	Tanzania	*Suleiman & Fundi 130A*	AY574222	—
**C. pilifer* 6	Rwanda	*Frahm 7611*	AY375235	—
**C. pilifer* 7	Zaire	*Pócs 6939*	AY375234	—

Table 1. Continued.

Taxon	Geographical origin	Voucher	GenBank accession No.	
			ITS1/5.8S/ITS2	*atp*B-*rbc*L spacer
**C. pilifer* 8	Socotra	*Kürschner 02-213*	AY372475	
**C. pilifer* 9	Socotra	*Kilian YP2538a*	AY372476	AY551372
**C. pilifer* 10	Saudi Arabia	*Kürschner 1-4071*	AY372477	—
**C. pilifer* 11	Cape Verde	*Lindlar 16*	AY372482	—
**C. pilifer* 12	Cape Verde	*Lindlar 458*	AY372481	AY576439
**C. pilifer* 13	Canary Islands	*Zippel 96-52.3*	AY375221	—
**C. pilifer* 14	Azores	*Wallace 1-3633*	AY375220	—
**C. pilifer* 15	Azores	*Schwab SN116*	AY372483	AY551371
**C. pilifer* 16	Azores	*Eggers Az 7,4*	AY372484	—
**C. pilifer* 17	Italy	23 Aug. 2000, *Frahm s.n.*	AY372478	AY576440
**C. pilifer* 18	Spain	*Muñoz 4060*	AY372480	—
**C. pilifer* 19	France	15 Mar. 2000, *Frahm s.n.*	AY372478	—
**C. pilifer* 20	Brazil	*Kalb & Poelt 1-3641*	AY375216	—
**C. pilifer* 21	Brazil	19 Jul. 1977, *Frahm s.n.*	AY375211	—
**C. pilifer* 22	Argentina	*Churchill & Schiavone 20076*	AY372470	AY576438
**C. pilifer* 23	Peru	*Weigend et al. 5832*	AY375213	—
**C. pilifer* 24	Peru	*Frahm 1-3590*	AY375214	—
**C. pilifer* 25	U.S.A., Georgia	*Frahm 1-3656*	AY375215	—
**C. pilifer* 26	Mexico	*Frahm & Eggers 1-3621*	AY574221	—
**C. pilifer* 27	U.S.A., Texas	*Merrill 13328*	AY372474	AY551368
**C. pilifer* 28	U.S.A., Arkansas	*Allen 6295*	AY372472	—
**C. pilifer* 29	Honduras	*Allen 11755*	AY372473	—
C. pilifer 30	Costa Rica	*Arts CR 21/12*	AY444135	AY159930
C. pilifer var. *lamellatus* (Mont.) Gradst. & Sipman	Peru	*Frahm 1-3589*	AY375212	AY159927
C. pyriformis (Schultz) Brid.	New Zealand	*Frahm X14-4*	AF444121	AY159916
C. robillardei Besch.	South Africa	*Arts RSA123/14*	AF444127	AY159912
C. savannarum (Müll. Hal.) Mitt.	Equatorial Guinea	*Heras 25.7.94, dupl. ex VIT449/94*	AF444125	AY159909
C. tallulensis Sull. & Lesq.	Colombia	*Lineares C. & Churchill 3820*	AF444118	AY159913
C. thwaitesii (Mitt.) A. Jaeger 1	South Africa	*Arts RSA 104/25*	AF444115	AY159906
C. thwaitesii 2	Brazil	*Vital & Buck 19682*	AF444116	AY159907
C. umbellatus (Schwägr. & Gaudich. ex Arn.) Paris	Indonesia	*Schäfer-Verwimp 20642*	AF444130	AY159928
OUTGROUP				
Dicranodontium denudatum (Brid.) E. Britton		June 1996, *Frahm s.n.*	—	AY159901
Leucobryum javense (Brid.) Mitt.		*Menzel et al. 381*	—	AF159900
L. juniperoideum (Brid.) Müll. Hal.		19 Aug. 1997, *Frahm s.n.*	—	AY159899
Pilopogon africanus Broth.		*Frahm 8079*	AF444139	AY159903
P. guadalupensis (Brid.) J.-P. Frahm		*Arts CR03/07*	AF444112	AY159904

Results

Alignments and sequence comparisons

Sequence lengths for the ITS1 region range approximately from 337 to 474 bp, those for the ITS2 region from 282 to 352 bp among all investigated *Campylopus* species. In *C. introflexus*, sequence lengths vary between 420 and 446 bp in the ITS1 region and between 293 and 343 bp in the ITS2 region. Sequence lengths in *C. pilifer* are 365–455 bp (ITS1) and 317–352 bp (ITS2). With 1659 positions, the ITS1 and ITS2 alignments were considerably longer than the sequences, due to numerous indels and particularly within the two types of the ITS1 (see discussion), which are in parts too divergent to be alignable and were therefore grouped into separate blocks in the alignment (Fig. 2). For tree construction, 1035 positions were used; 259 (25.0%) of them are variable, and 176 of the variable characters (68.0%, or 17.0% of the total number of characters) are parsimony-informative.

Lengths of the *atp*B-*rbc*L spacer range from 455 to 535 bp in the investigated *Campylopus* species (516–519 bp in *C. introflexus* and 521–535 bp in *C. pilifer*). The alignment comprises 61 positions of the 3′ end of the *atp*B gene and 595 positions belonging to the spacer, which were all used for tree construction. Of the 656 positions, 80 (12.2%) are variable, and 41 of the variable characters (51.3%, or 6.3% of the total number of characters) are parsimony-informative. Of the parsimony-uninformative positions, 21 represent autapomorphies for the outgroup taxa (*Dicranodontium* and *Leucobryum*), and 16 are autapomorphies for species of *Campylopus* and *Pilopogon*.

Phylogenetic analyses

Due to the almost identical sequences within *Campylopus introflexus* and *C. pilifer* from the Old World, high numbers of equally most parsimonious trees (MPTs) were found. Strict consensus trees including all investigated specimens are calculated from 100,000 saved MPTs (ITS1 and ITS2: lengths 414 steps, CI [excluding uninformative characters] = 0.666, RI = 0.907, RC = 0.668; ITS1 only: lengths 174, CI = 0.796, RI = 0.957, RC = 0.809; ITS2 only: lengths 224, CI = 0.629, RI = 0.876, RC = 0.618; *atp*B-*rbc*L spacer: lengths 98, CI = 0.821, RI = 0.896, RC = 0.804). Only in the combined analysis of ITS1, ITS2, and *atp*B-*rbc*L spacer of 35 *Campylopus* specimens for which all three DNA regions were sequenced, a smaller number of 104 MPTs (lengths 405, CI = 0.700, RI = 0.864, RC = 0.670) were recovered. To allow for more thorough searches, parsimony analyses of the ITS regions were repeated with only two accessions of *C. introflexus* (1 and 6) and five accessions of *C. pilifer* from the Old World (*C. pilifer* 1, 4, 8, 11, and 17), i.e., most of the identical sequences were excluded. These searches resulted in 810 MPTs for ITS1 and ITS2 (lengths 386 steps, CI [excluding uninformative characters] = 0.667, RI = 0.855, RC = 0.631) and 7923 MPTs for ITS2 only (lengths 210, CI = 0.627, RI = 0.811, RC = 0.572), whereas for ITS1 again 100,000 trees were found (lengths 183, CI = 0.741, RI = 0.911, RC = 0.732).

In the ITS1 and ITS2 tree of all specimens (Fig. 3), *Campylopus pilifer* and *C. introflexus* together are separated from the other species in a clade with only 52% bootstrap support (BS) and a decay index (DI) of 2. Within this clade, three well-supported subclades are observed. The first one comprises all samples of *C. introflexus* (99% BS, DI = 5). The second clade holds all Central American collections of *C. pilifer* plus two North American populations (96% BS, DI = 5). All but one South American samples of *C. pilifer* compose, with a population from Georgia, the third clade (100% BS, DI = 7). The African, Macaronesian, Arabian, and European samples and one specimen from Brazil are poorly differentiated from each other and hence of ambiguous affinity. In a maximum likelihood analysis of the same data set, 29 trees with almost identical topologies were recovered (ln L = −3758.3781), of which one tree is shown in Figure 4. As in the parsimony tree, all specimens of *C. pilifer* and *C. introflexus* come together on one unsupported branch, but a slightly higher resolution between the Old World specimens of *C. pilifer* is achieved, and *C. introflexus* (99% BS) is nested within the main branch of the African, Macaronesian, Arabian, and European *C. pilifer* (77% BS). When analyzing only a selection of specimens, a sister group relationship between the clades of *C. pilifer* 8, 11, and 17 (Italy, Cape Verde, Socotra) and of *C. introflexus* is indicated (91% BS), similar to the ML tree, whereas relationships of *C. pilifer* 1 (South Africa), 4 (Réunion), and 20 (Brazil) remain ambiguous (see inset in Fig. 3). Otherwise the topology is identical to the tree comprising all accessions.

Separate analyses of ITS1 and ITS2 result in trees with different topologies. In the ITS1 tree (Fig. 5), a closer relationship of *Campylopus introflexus* and most of the Old World *C. pilifer* samples is indicated, similar to the maximum likelihood analysis of ITS1 and ITS2. The respective specimens form a basal polytomy separated from a clade of the other taxa (64% BS, DI = 1), including all Amer-

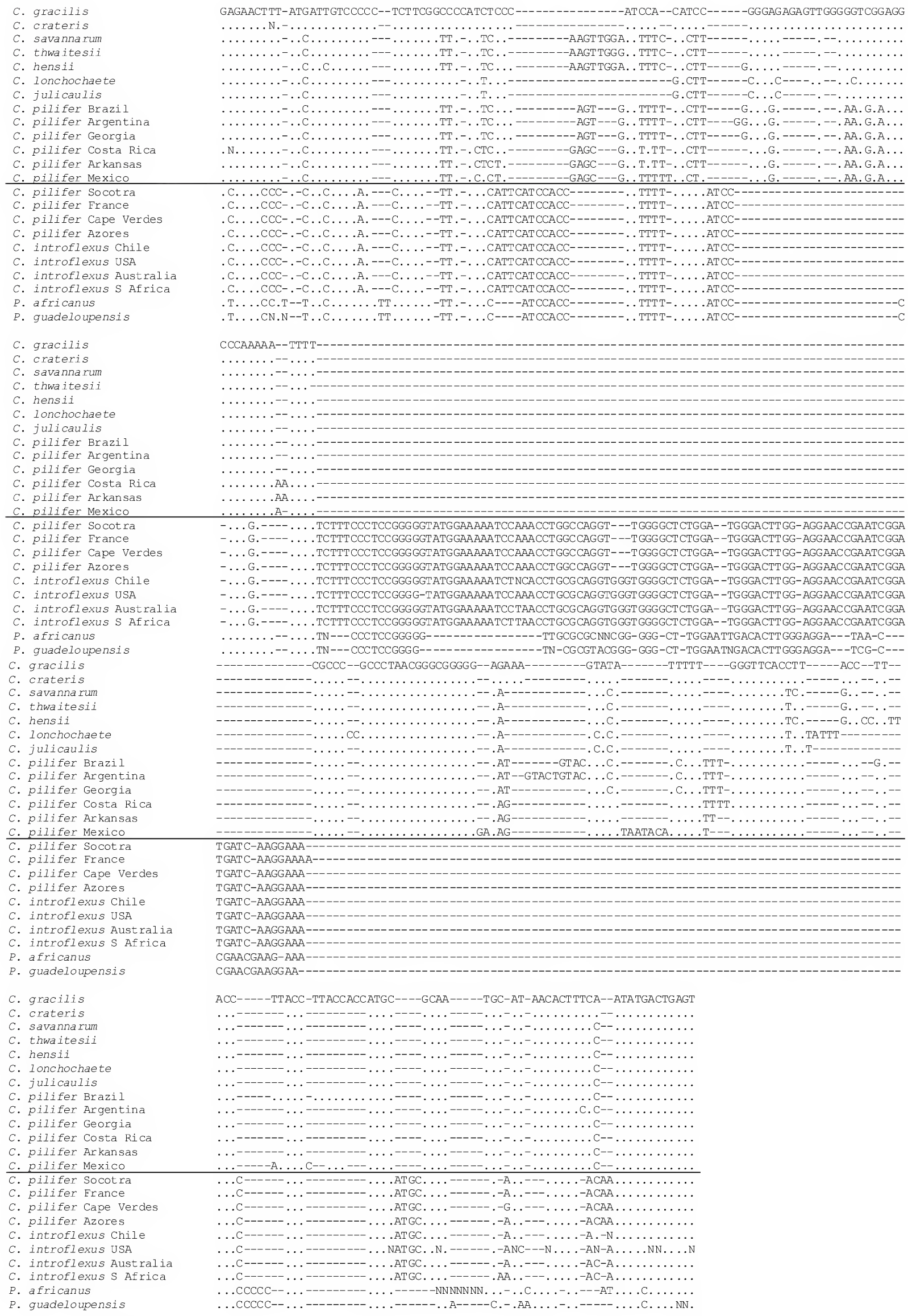
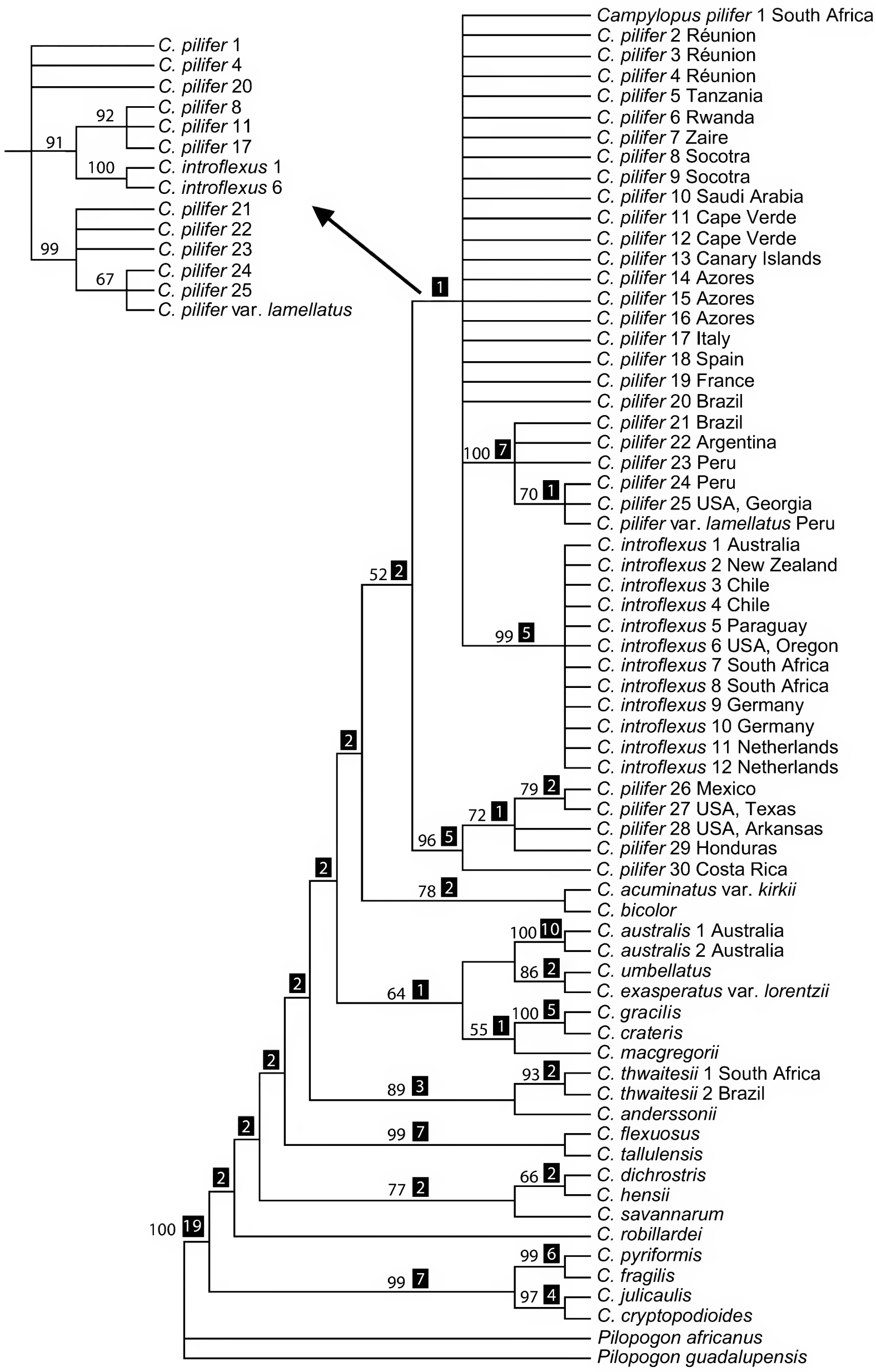


Figure 2. Detail of the ITS1 alignment of selected sequences displaying the two different sequence types, type I (above the line) and type II (below the line).


C. pilifer 1
C. pilifer 4
C. pilifer 20
C. pilifer 8
C. pilifer 11
C. pilifer 17
C. introflexus 1
C. introflexus 6
C. pilifer 21
C. pilifer 22
C. pilifer 23
C. pilifer 24
C. pilifer 25
C. pilifer var. lamellatus
92
91
100
99
67
Campylopus pilifer 1 South Africa
C. pilifer 2 Réunion
C. pilifer 3 Réunion
C. pilifer 4 Réunion
C. pilifer 5 Tanzania
C. pilifer 6 Rwanda
C. pilifer 7 Zaire
C. pilifer 8 Socotra
C. pilifer 9 Socotra
C. pilifer 10 Saudi Arabia
C. pilifer 11 Cape Verde
C. pilifer 12 Cape Verde
C. pilifer 13 Canary Islands
C. pilifer 14 Azores
C. pilifer 15 Azores
C. pilifer 16 Azores
C. pilifer 17 Italy
C. pilifer 18 Spain
C. pilifer 19 France
C. pilifer 20 Brazil
C. pilifer 21 Brazil
C. pilifer 22 Argentina
C. pilifer 23 Peru
C. pilifer 24 Peru
C. pilifer 25 USA, Georgia
C. pilifer var. lamellatus Peru
C. introflexus 1 Australia
C. introflexus 2 New Zealand
C. introflexus 3 Chile
C. introflexus 4 Chile
C. introflexus 5 Paraguay
C. introflexus 6 USA, Oregon
C. introflexus 7 South Africa
C. introflexus 8 South Africa
C. introflexus 9 Germany
C. introflexus 10 Germany
C. introflexus 11 Netherlands
C. introflexus 12 Netherlands
C. pilifer 26 Mexico
C. pilifer 27 USA, Texas
C. pilifer 28 USA, Arkansas
C. pilifer 29 Honduras
C. pilifer 30 Costa Rica
C. acuminatus var. kirkii
C. bicolor
C. australis 1 Australia
C. australis 2 Australia
C. umbellatus
C. exasperatus var. lorentzii
C. gracilis
C. crateris
C. macgregorii
C. thwaitesii 1 South Africa
C. thwaitesii 2 Brazil
C. anderssonii
C. flexuosus
C. tallulensis
C. dichrostris
C. hensii
C. savannarum
C. robillardei
C. pyriformis
C. fragilis
C. julicaulis
C. cryptopodioides
Pilopogon africanus
Pilopogon guadalupensis
1
52 2
2
2
2
2
2
100 19
100 7
70 1
99 5
79 2
72 1
96 5
78 2
100 10
86 2
64 1
100 5
55 1
93 2
89 3
99 7
66 2
77 2
99 6
99 7
97 4

ican *C. pilifer* specimens and the specimens from South Africa and Réunion. In the analysis of a reduced number of specimens (tree not shown), a well-supported clade (99% BS) replaces this basal polytomy. In the ITS2 analyses the Old World *C. pilifer* samples except for the South African one appear on one branch, either without significant support (tree of all specimens, Fig. 6) or with 67% BS (tree comprising only the selected specimens, not shown); relationships to *C. introflexus* are ambiguous. As in the ITS1 and ITS2 analysis, high support is gained for the clades of *C. introflexus* (95% BS, DI = 9), the Central and North American *C. pilifer* samples (90% BS, DI = 5), and the South American samples (99% BS, DI = 6).

The maximum likelihood analysis of the *atp*B-*rbc*L spacer sequences resulted in a single tree (ln L = −1348.9008; Fig. 7) that is almost identical to the strict consensus tree based on maximum parsimony (not shown). Two branches are observed in the likelihood tree only (indicated by arrows in Fig. 7), whereas in the parsimony tree the respective specimens are part of a large polytomy with other investigated *Campylopus* species. The clades of *Campylopus introflexus* (MP: 83% BS, DI = 1; ML: 79% BS) and the Old World *C. pilifer* specimens (MP: 66% BS, DI = 1; ML: 60% BS) are found again. In contrast to the ITS analyses, however, the American *C. pilifer* specimens are part of the large polytomy, except for both South American samples in the ML tree (61% BS), and a close relationship of *C. introflexus* with *C. crateris* and *C. gracilis* is indicated.

Finally, combined analyses of ITS1, ITS2, and *atp*B-*rbc*L spacer support a sister group relationship of *Campylopus introflexus* and the Old World *C. pilifer* specimens with bootstrap values of 99% in the parsimony consensus tree (not shown) and in the single optimal maximum likelihood tree (ln L = −4676.7386; Fig. 8). These results are in accordance with the ML analysis of ITS1 and ITS2 (Fig. 4). A clade comprising the American *C. pilifer* specimens yields 59% BS (MP: 77%) and is resolved as sister to *C. introflexus* and the Old World *C. pilifer*.

Discussion

Campylopus introflexus is monophyletic with 95%–99% bootstrap support in the trees based on ITS1 and ITS2 (Figs. 3, 4), ITS2 only (Fig. 6), and combined ITS + *atp*B-*rbc*L spacer (Fig. 8). The monophyly of *C. introflexus* is further supported by the analysis of the *atp*B-*rbc*L spacer only (Fig. 7), although the respective clade also includes *C. crateris* and *C. gracilis*. However, a close relationship between these species and *C. introflexus* is contradicted by morphological and molecular characters. Morphologically, *C. gracilis* much resembles *C. pyriformis* (Frahm, 1992), and is distinguished from *C. introflexus*, e.g., by the lack of hyaline hairpoints and of lamellae on the dorsal costa surface. *Campylopus crateris* differs from *C. introflexus* not only gametophytically (e.g., costa with ventral and dorsal stereids, without dorsal lamellae), but also by sporophytic characters, namely, the long filiform peristome teeth and finely papillose spores typical for *Campylopus* subg. *Thysanomitrion* (Frahm, 1985). At the molecular level, both species differ from *C. introflexus* not only by their ITS sequences, but also by a large deletion in the middle part of the *atp*B-*rbc*L spacer. This deletion comprises the region of a putative dyad-symmetrical element (hairpin secondary structure), which is present in most of the *Campylopus* species investigated in Stech (2004) and also in *C. introflexus* as revealed in the present study.

In contrast to *Campylopus introflexus, C. pilifer* appears as paraphyletic or polyphyletic in all molecular trees. The paraphyletic arrangement in the trees based on ITS1 and ITS2 (Figs. 3, 4) and combined ITS and *atp*B-*rbc*L spacer (Fig. 8), with *C. introflexus* nested within a clade of all *C. pilifer* specimens, supports the close relationship of both species and indicates that *C. introflexus* might be derived from *C. pilifer*. However, the low resolution of trees based on single markers (ITS1, ITS2, or *atp*B-*rbc*L spacer regions, respectively) and the missing statistical support for internal branches do not allow definite conclusions.

The degree of molecular variation in the ITS sequences differs considerably between *Campylopus introflexus* and *C. pilifer*. Similar to the lower morphological variation observed in *C. introflexus* compared to *C. pilifer* (Gradstein & Sipman, 1978), the ITS sequences exhibit only low variation in *C. introflexus*. This may be due to either low rates of evolution, or higher genetic exchange by long-range

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Figure 3. Strict consensus tree of 100,000 most parsimonious trees inferred from a heuristic search of combined ITS1 and ITS2 sequences of *Campylopus*, with *Pilopogon* as outgroup representative. Bootstrap values > 50% and decay indices (on a black background) are depicted above the branches. The inset shows a part of an otherwise identical tree including only a selection of specimens of *C. introflexus* and *C. pilifer* from the Old World.

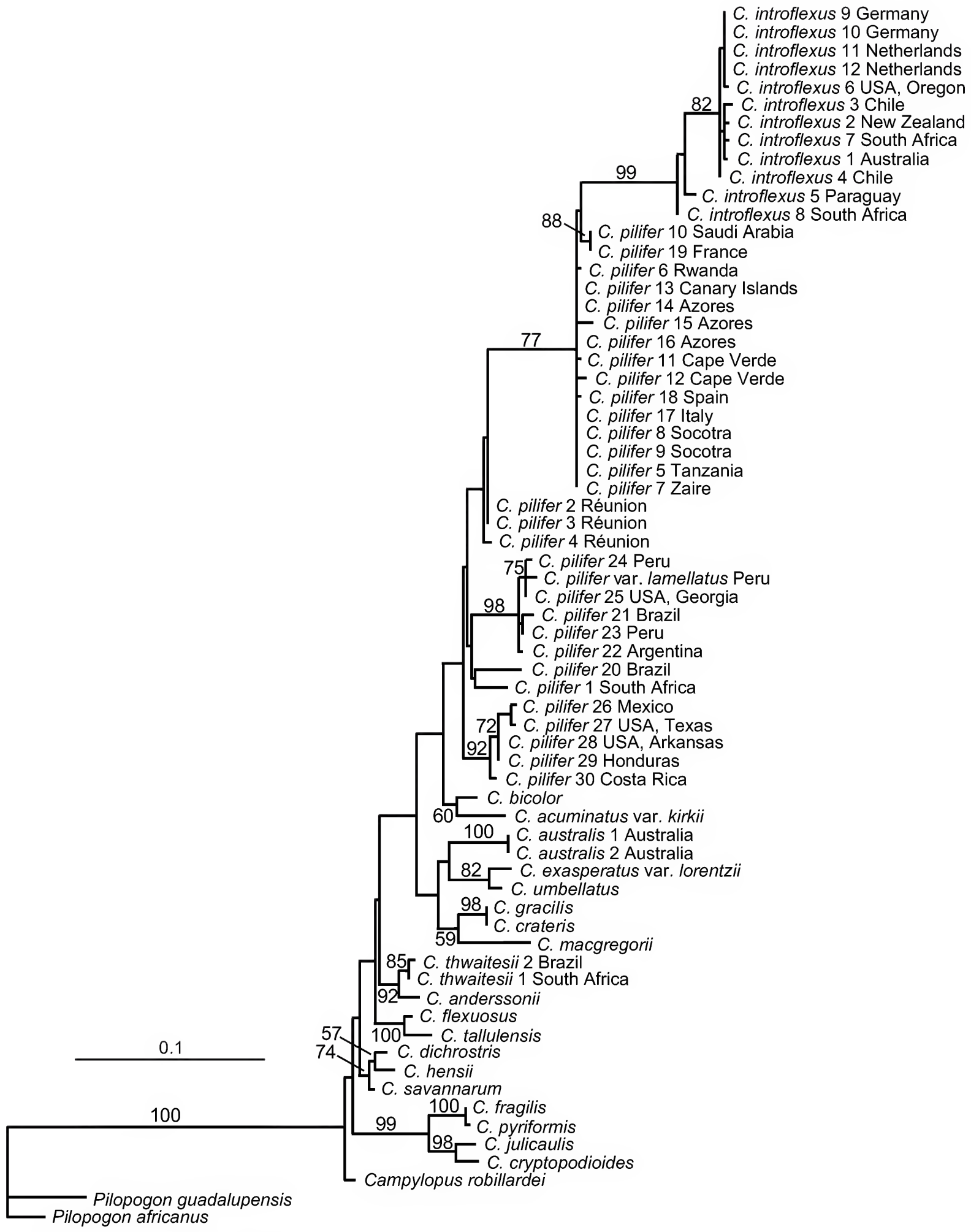

Figure 4. One of 29 trees obtained by a maximum likelihood analysis (GTR + G + I model) of combined ITS1 and ITS2 sequences of *Campylopus*, with *Pilopogon* as outgroup representative. Bootstrap values > 50% are given above the branches.

dispersal between *C. introflexus* populations in the temperate regions of the Southern Hemisphere than between *C. pilifer* populations in the tropics, facilitated by the prevailing westerly winds (van Zanten, 1978) and the higher frequency of sporophytes in *C. introflexus* (Gradstein & Sipman, 1978). Unfortunately, the low resolution in the ITS and *atp*B-*rbc*L spacer trees (Figs. 3–8) makes it impossible

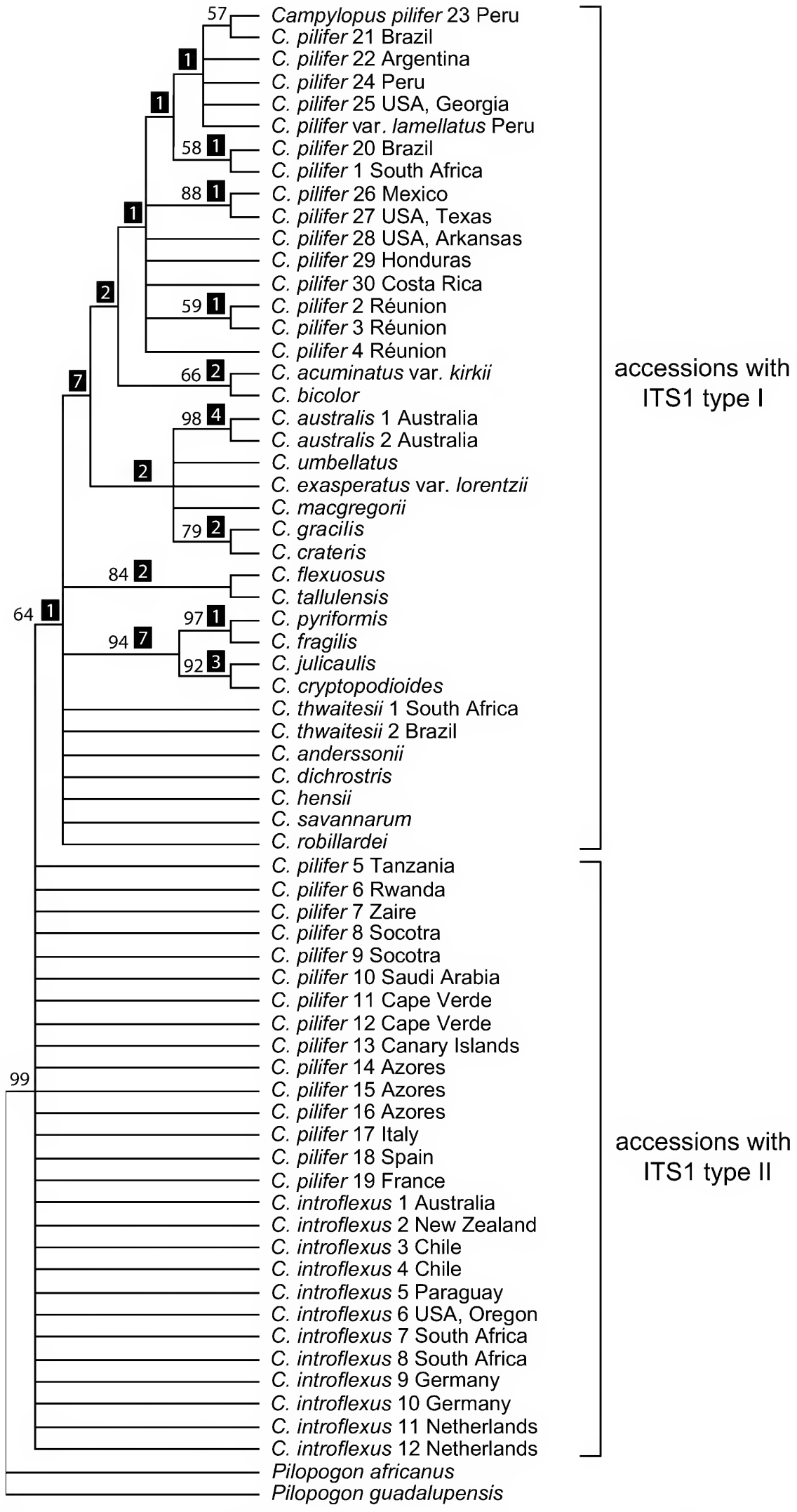

Figure 5. Strict consensus tree of 100,000 most parsimonious trees inferred from ITS1 sequences only. Bootstrap values $> 50\%$ and decay indices (on a black background) are depicted above the branches. Brackets indicate the distribution of the ITS1 type I and type II among the sequenced *Campylopus* specimens.

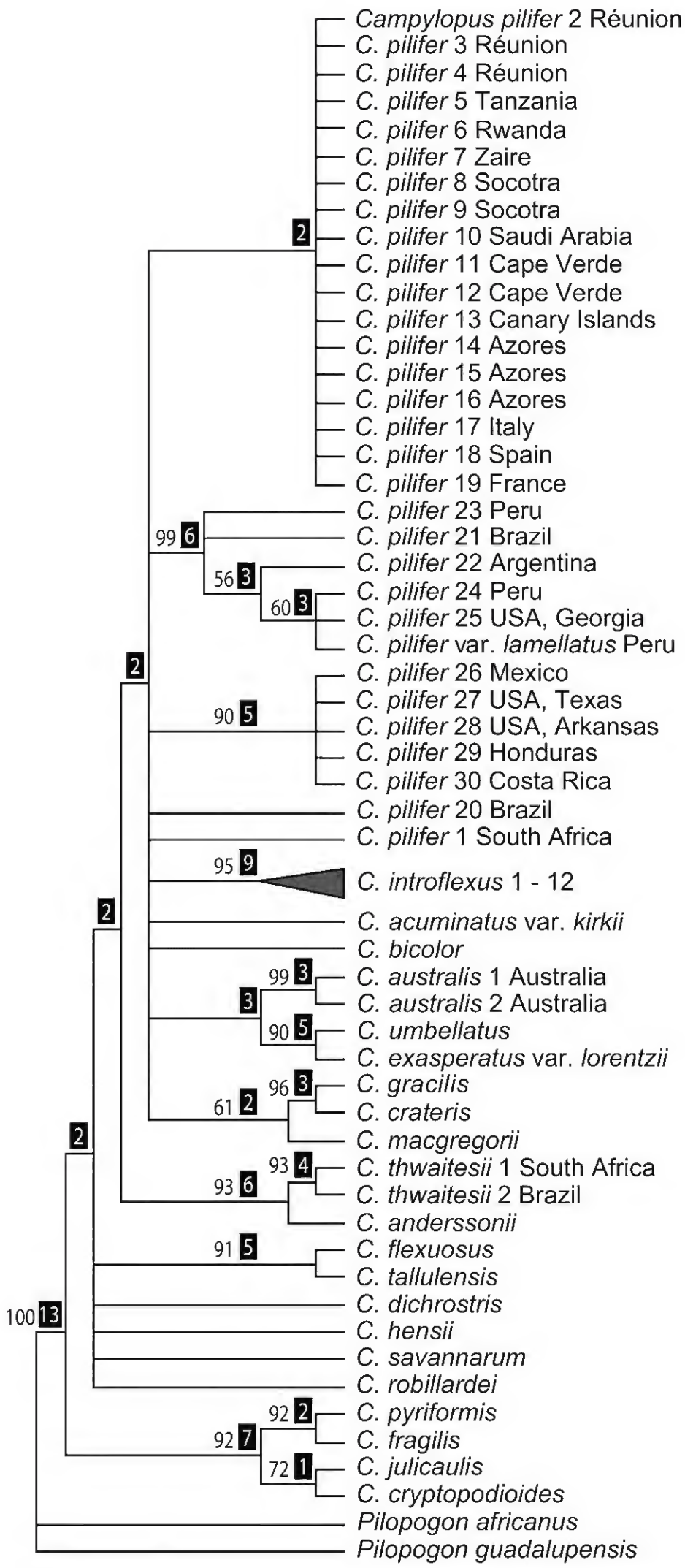

Figure 6. Strict consensus tree of 100,000 most parsimonious trees inferred from ITS2 sequences only. Bootstrap values > 50% and decay indices (on a black background) are depicted above the branches.

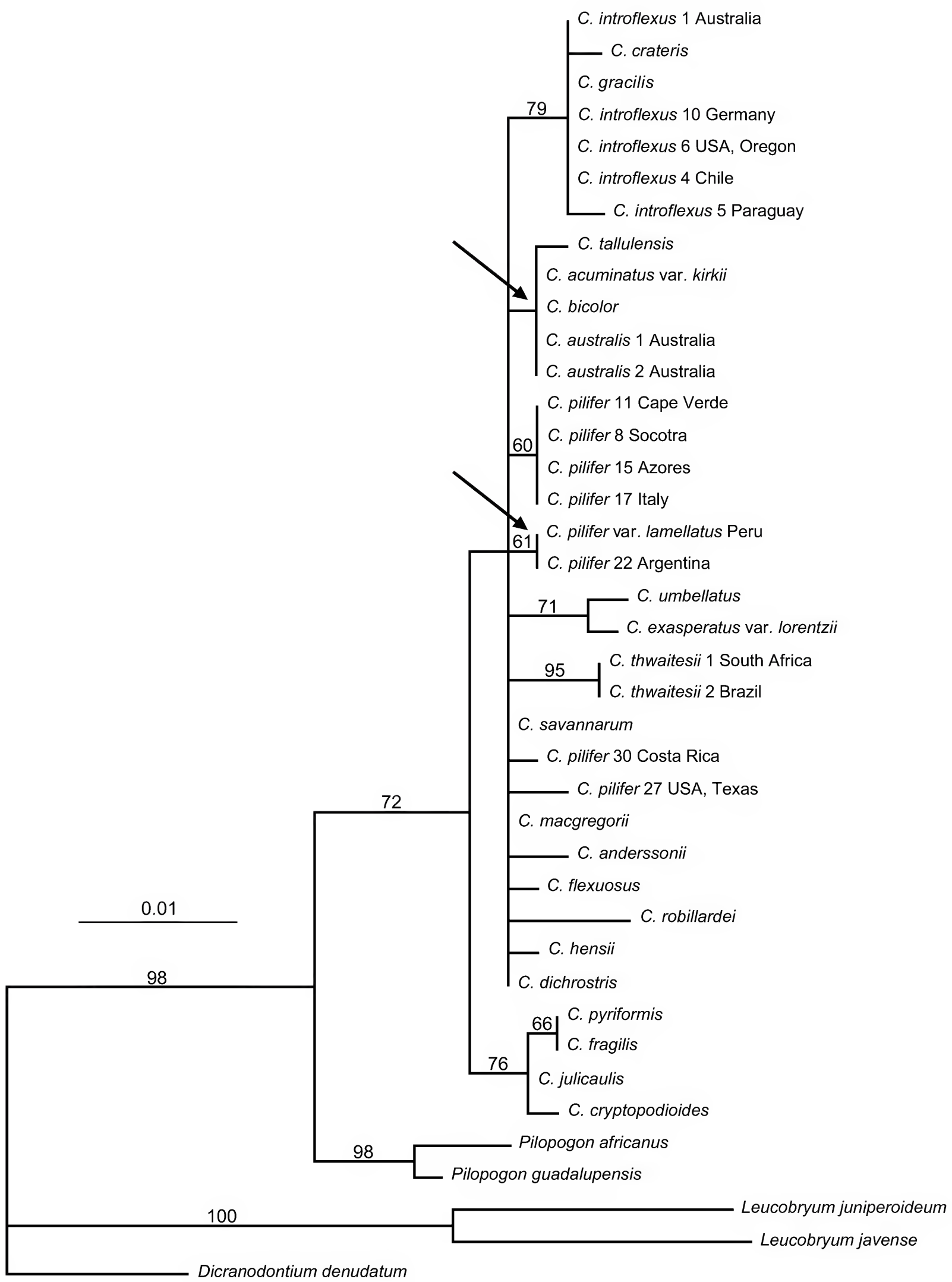

Figure 7. Single tree obtained by a maximum likelihood analysis (GTR + G model) of *atp*B-*rbc*L spacer sequences of *Campylopus* and *Pilopogon*, with *Dicranodontium* and *Leucobryum* as outgroup representatives. Bootstrap values > 50% are depicted above the branches. Arrows indicate branches that do not appear in an otherwise identical strict consensus tree of 100,000 trees obtained by maximum parsimony analysis.

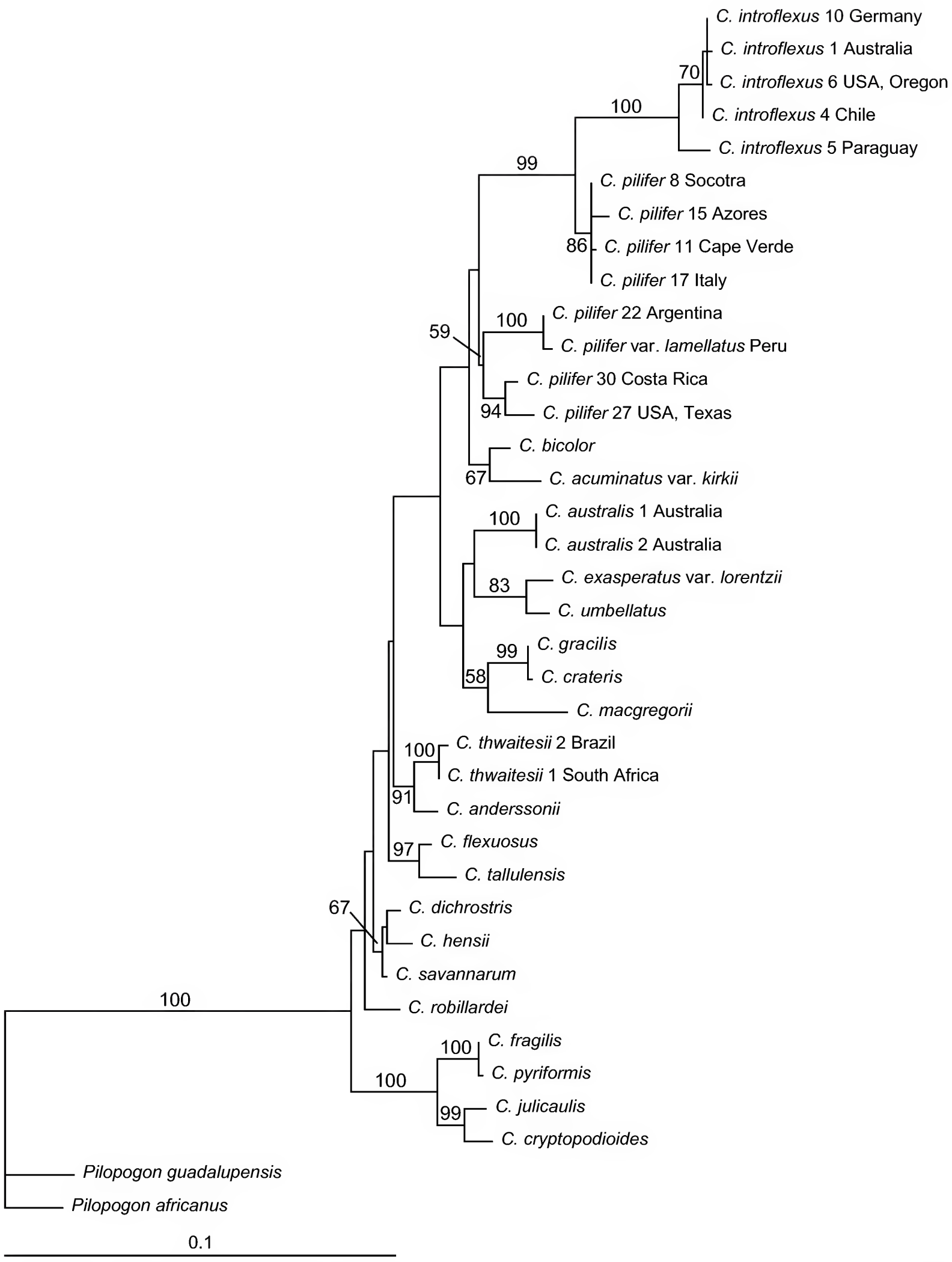

Figure 8. Single tree obtained by a maximum likelihood analysis (GTR + G + I model) of combined ITS1, ITS2, and *atp*B-*rbc*L spacer sequences of *Campylopus*, with *Pilopogon* as outgroup representative. Bootstrap values > 50% are depicted above the branches.

to detect possible infraspecific biogeographical patterns and to infer the origin of the populations introduced into North America and Europe.

Campylopus pilifer is morphologically and molecularly more variable than *C. introflexus*. Indicators of the morphological variation are numerous synonyms (cf. Frahm, 1999) and the description of infraspecific taxa. At present, *C. pilifer* is divided into three subspecies, subspecies *galapagensis* (J.-P. Frahm & Sipman) J.-P. Frahm, subspecies *vaporarius* (De Not.) Brullo, Privitera & M. Puglisi, and subspecies *pilifer*. The subspecies *galapagensis* is characterized by ventral substereids instead of hyalocysts in the costa (Frahm, 1987), while subspecies *vaporarius* represents a large and robust taxon confined to fumaroles of volcanic areas in the Mediterranean region (Brullo et al., 2001). In addition, *C. pilifer* subsp. *pilifer* var. *lamellatus* (Mont.) Gradst. & Sipman was separated from variety *pilifer* because of higher lamellae of 5 to 7 cells on the dorsal costa surface (Gradstein & Sipman, 1978). Finally, *C. pilifer* fo. *angustifolius* (Warnst.) J.-P. Frahm was assumed to be a possible hybrid with *C. introflexus* (Frahm, 1979). All infraspecific taxa of *C. pilifer* were earlier recognized as separate species (cf. Frahm, 1999). The ITS data clearly confirm the inclusion of *C. lamellatus* Mont. (Montagne, 1838) in *C. pilifer*, as done by Gradstein and Sipman (1978). Furthermore, the close molecular relationship of the specimen of variety *lamellatus* from Peru to other South American samples belonging to variety *pilifer* (Figs. 3, 4) indicates that the number of cells of the dorsal lamellae might not be a useful taxonomic character, and that the respective specimen may rather be regarded as an extreme form of variety *pilifer*.

Our analyses reveal a geographical pattern in the haplotype distribution within *Campylopus pilifer*, namely, a basic division into an Old World and one or two New World lineages. A clade comprising the Arabian, Macaronesian, European, and most of the African specimens is observed in the ITS2 tree (Fig. 6) and in the maximum likelihood tree of ITS1 and ITS2 (Fig. 4). Although bootstrap support is lacking and a polytomy of these specimens occurs in the remaining ITS analyses (Figs. 3, 5), the existence of an Old World group is supported by a respective clade in the trees calculated from the *atp*B-*rbc*L spacer (Fig. 7, 60% BS) and from combined nrDNA + cpDNA sequences (Fig. 8, 86% BS). Most of the American specimens are divided into two well-supported clades the ITS1 and ITS2 trees (Figs. 3, 4) and in the tree based on ITS2 only (Fig. 6), a South American clade (which also includes the specimen from Georgia, U.S.A.) and a clade comprising the remaining Central/North American specimens. Only in the ITS1 tree (Fig. 5) are all New World specimens placed together in one clade without statistical support.

This basic division into Old World and New World lineages probably reflects divergent evolution of separated populations after the break up of Gondwana. Interestingly, the molecular variation is higher in the New World, which was also observed in the three species analyzed by Shaw et al. (2003), *Claopodium whippleanum* (Sull.) Ren., *Dicranoweisia cirrata* (Hedw.) Lindb., and *Scleropodium touretii* (Brid.) L. F. Koch. The Northern Hemispheric populations of *C. pilifer* seem to be of two different origins, and migrated from South America to North America and from Africa to Macaronesia and Europe. Although geological processes might have played an important role in the formation of infraspecific evolutionary lineages, long-range dispersal of spores or introduction events can also be assumed. Examples are the specimen from U.S.A. (Georgia) and the samples from South Africa and Réunion, which seem to be more closely related to the New World specimens (Figs. 4, 5) than to the other Old World specimens.

Both cases of either high (as in *Campylopus pilifer*) or low sequence variation (as in *C. introflexus*) have already been observed in the internal transcribed spacers of other bryophyte species. For example, *Fontinalis antipyretica* Hedw. (Shaw & Allen, 2000), *Mielichhoferia elongata* (Hoppe & Hornsch.) Nees & Hornsch. and *M. mielichhoferiana* (Funck) Loeske (Shaw, 2000) and *Hygrohypnum smithii* (Sw.) Broth. (Stech & Frahm, 2001) were revealed as paraphyletic or polyphyletic, respectively. Apart from *Fontinalis*, *Mielichhoferia*, and the three genera analyzed by Shaw et al. (2003), geographical patterns were also found in *Hylocomium splendens* Hedw. (Chiang & Schaal, 1999) and *Symphyogyna hymenophyllum* (Hook.) Mont. & Nees/*S. podophylla* (Thunb.) Mont. & Nees (Schaumann et al., 2003) based on ITS2 sequences. In contrast, low sequence variation was observed in *Anacolia menziesii* (Turner) Parihar/*A. webbii* (Mont.) Schimp. (Werner et al., 2003), *Thamnobryum alopecurum* (Stech et al., 2001), and *Weymouthia cochlearifolia* (Schwaegr.) Dix./*W. mollis* (Hedw.) Broth. (Quandt et al., 2001).

The striking phenomenon of different ITS types in one genus, however, is reported here for the first time in bryophytes. While the ITS2 sequence is, despite considerable length variation, similar in all *Campylopus* species, in the ITS1 sequence two very different, partly unalignable types exist (Fig. 2), one in *Campylopus introflexus* and the Old World *C.*

pilifer specimens (here tentatively named "type II"), and the second in the New World *C. pilifer* specimens and in the other investigated *Campylopus* species ("type I") (Fig. 5). Moreover, the type II is more similar to the ITS1 of *Pilopogon* than to the type I (Fig. 2). Several explanations for these findings are conceivable. Considering only *Campylopus*, the existence of ITS1 paralogues might be suspected at first sight; however, this seems unlikely due to the clear systematic and (within *C. pilifer*) geographical distribution patterns of both ITS1 types. Another possibility is that the type II derived from an inversion of type I; this, however, is contradicted by three different findings: (1) an artificial inversion of the type II in the alignment program did not reveal any similarity with type I, (2) short sequence motifs occurring in both types are no longer present in the artificial inversion of type II, and (3) long C/T and A/G repeat segments near the 3′ end in type II have no counterpart in type I. The occurrence of the same sequence type in *C. introflexus* and the Old World *C. pilifer* specimens might indicate hybridization between both species (as supposed, e.g., by Gradstein & Sipman, 1978), but as almost all investigated extant populations of *C. pilifer* are affected, an ancient hybridization event instead of multiple independent events in more recent times must be assumed, which is not supported by other data. Therefore, the most plausible explanation is that *C. pilifer* is paraphyletic, in agreement with the topologies of the maximum likelihood trees shown in Figures 4 and 8, with the different lineages representing cryptic species. If the type II is homologous to the ITS1 of *Pilopogon*, it would represent the plesiomorphic type and not a synapomorphy of *C. introflexus* and the Old World *C. pilifer*. This question cannot be decided at present, although the clear separation of *Pilopogon* and *Campylopus* by the ITS2 and chloroplast markers might rather indicate an independent evolution of the ITS1 in both genera.

Different ITS types have so far been reported only rarely from angiosperms. For example, in Winteraceae, two types occur in the genera *Bubbia* Tiegh., *Exospermum* Tiegh., and *Zygogynum* Baill. (Suh et al., 1993), but these types are distinguished mainly by substitutions in the ITS1 and ITS2, in contrast to the extensive length and sequence variation in *Campylopus* only in the ITS1. Two very different sequence types were recently also found in the ITS of Calycanthaceae (Jianhua et al., 2003), which may perhaps correspond to the situation in *Campylopus*.

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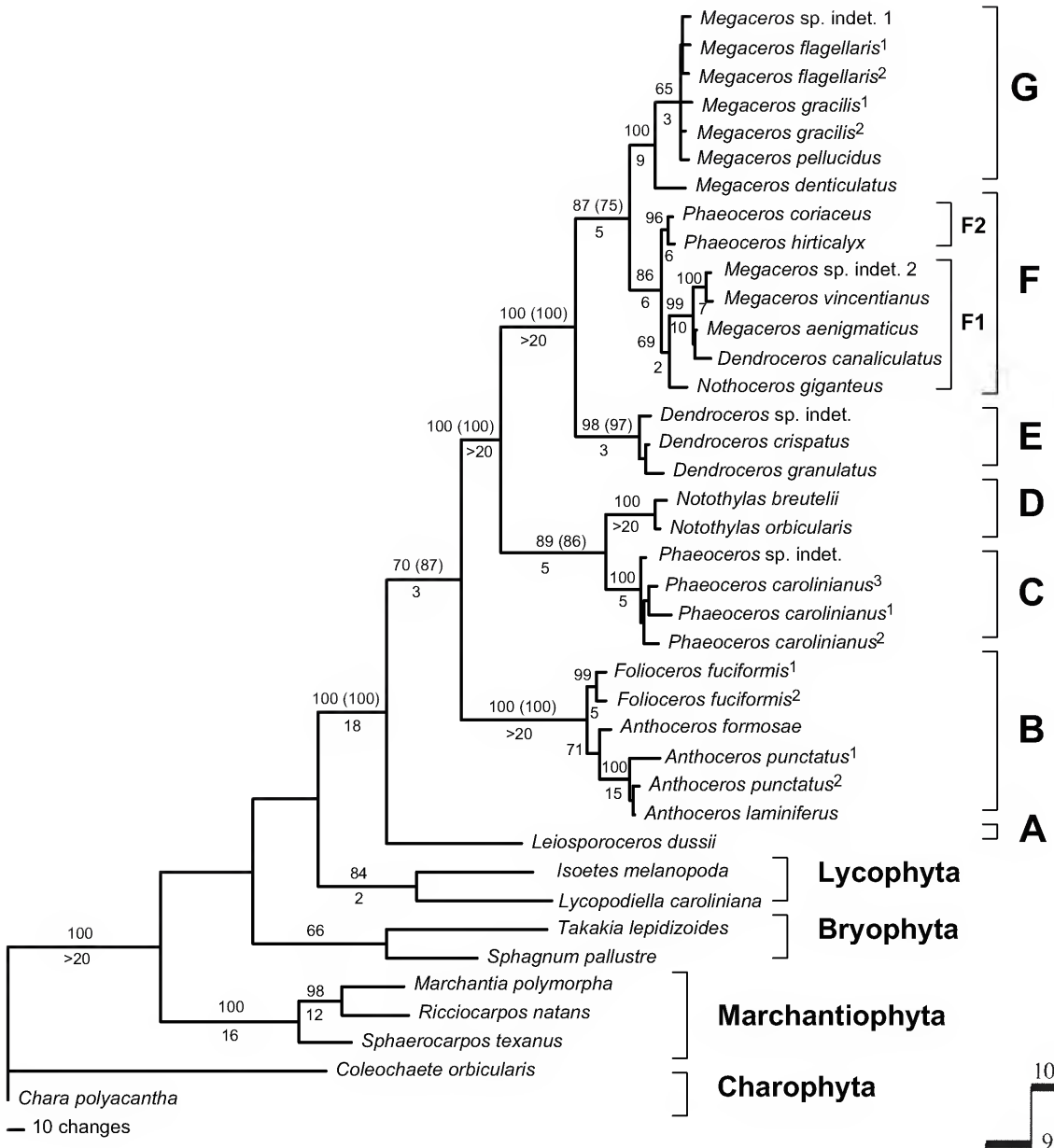
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Phaeoceros hirticalyx
Megaceros sp. indet. 2
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Megaceros aenigmaticus
Dendroceros canaliculatus
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Dendroceros granulatus
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Notothylas orbicularis
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Folioceros fuciformis2
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Anthoceros punctatus2
Anthoceros laminiferus
Leiosporoceros dussii
Isoetes melanopoda
Lycopodiella caroliniana
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Sphagnum pallustre
Marchantia polymorpha
Ricciocarpos natans
Sphaerocarpos texanus
Coleochaete orbicularis
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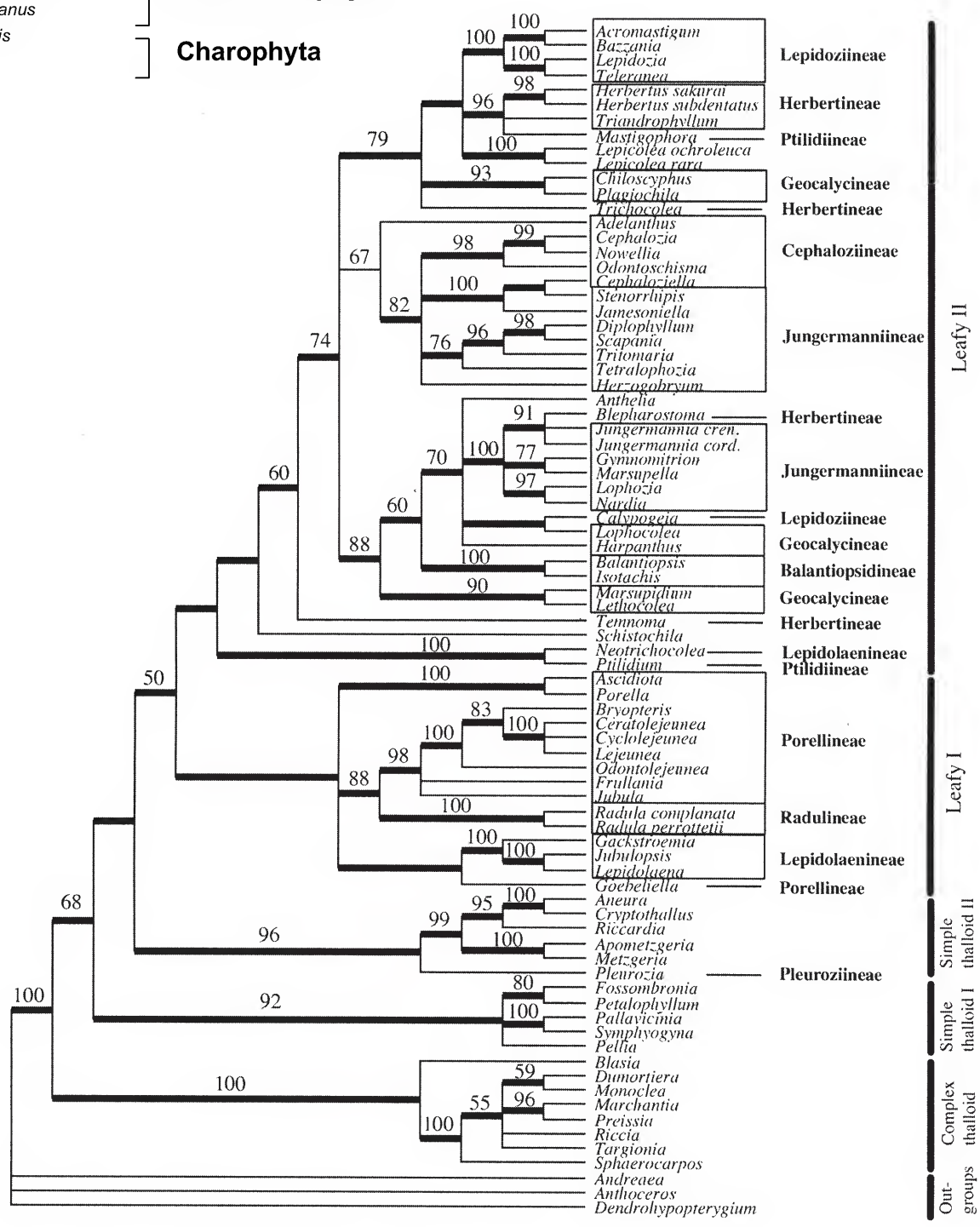
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Nowellia
Odontoschisma
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Stenorrhipis
Jamesoniella
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Scapania
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Anthelia
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Jungermannia cren.
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Lepidolaena
Goebelella
Aneura
Cryptothallus
Riccardia
Apometzgeria
Metzgeria
Pleurozia
Fossombronia
Petalophyllum
Pallavicinia
Symphyogyna
Pellia
Blasia
Dumortiera
Monoclea
Marchantia
Preissia
Riccia
Targionia
Sphaerocarpos
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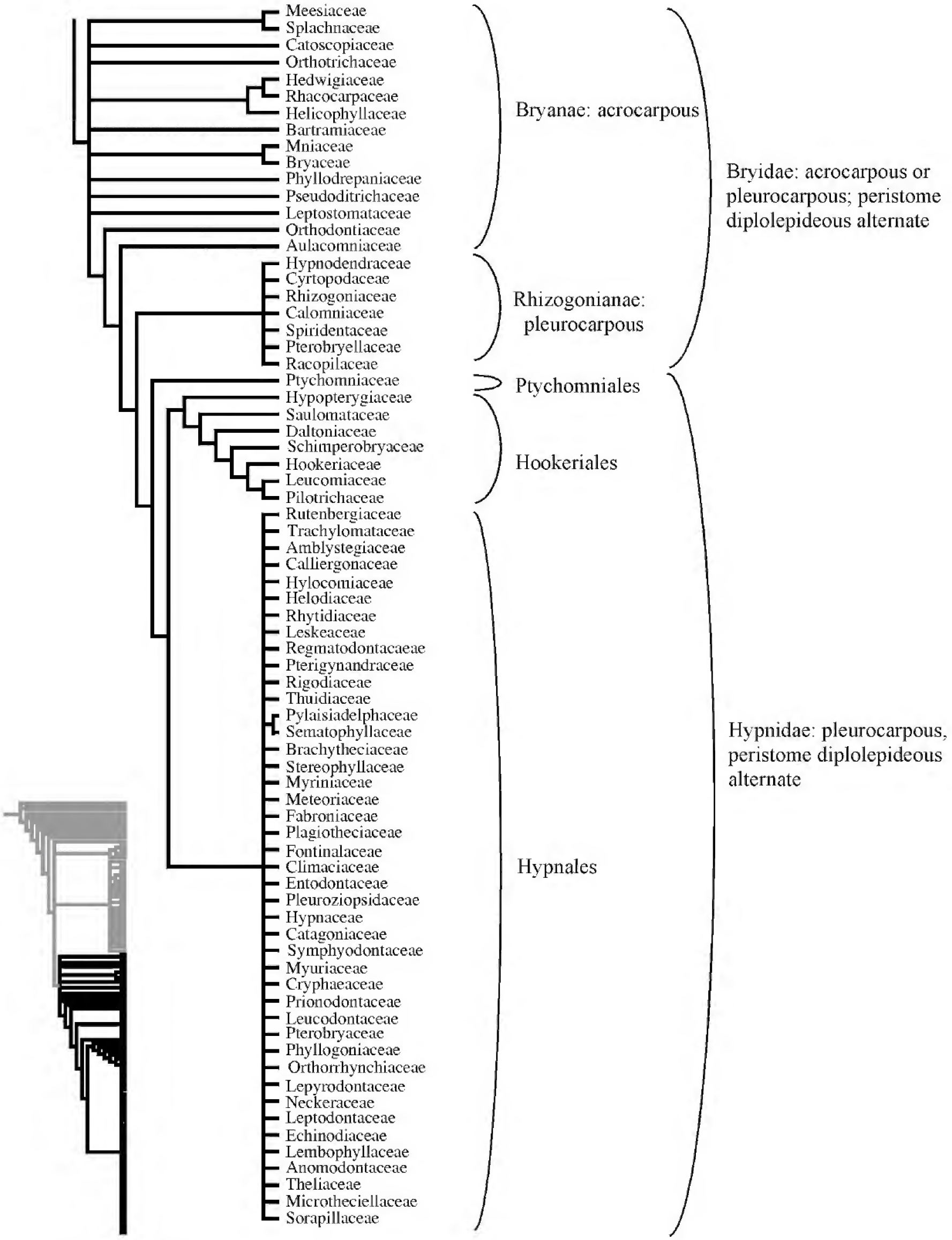
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Fontinalaceae
Climaciaceae
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Hypnaceae
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Orthorrhynchiaceae
Lepyrodontaceae
Neckeraceae
Leptodontaceae
Echinodiaceae
Lembophyllaceae
Anomodontaceae
Theliaceae
Microtheciellaceae
Sorapillaceae
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Rhizogonianae: pleurocarpous
Ptychomniales
Hookeriales
Hypnales
Bryidae: acrocarpous or pleurocarpous; peristome diplolepideous alternate
Hypnidae: pleurocarpous, peristome diplolepideous alternate

Molecular Systematics of Bryophytes: Progress, Problems and Perspectives was an internationally attended symposium held at the Missouri Botanical Garden, September 6 and 7, 2003. The conference was organized by Bernard Goffinet from the University of Connecticut and Robert Magill, Director of Research at the Missouri Botanical Garden, with sponsorship from the Garden and the Deep Gene Research Coordination Network. Attendees from a dozen countries, representing a global bryological community, participated in the event.

The symposium program featured twenty-nine speakers discussing various aspects of the molecular systematics of bryophytes (mosses, liverworts, and hornworts). The result of this gathering of imminent botanical researchers is the book now before you, twenty-one chapters divided into five areas of concentration: (1) Bryophytes and Evolution of Land Plants; (2) Hornwort Phylogeny; (3) Liverwort Phylogeny; (4) Moss Phylogeny; and (5) Phylogeography.

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